THE OSMOTIC PROPERTIES OF HUMAN ERYTHROCYTES AND BODY FLUIDS.

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by

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A simple accurate method is described for determining the degree of haemolysis in a system containing lysed erythrocytes. It has been shown that haemolysis is an all-or-none phenomenon.

It is known that oxygenation decreases the fragility of erythrocytes, and it has been proved that this is due to an increase in pH resulting from the removal of carbon dioxide. The effect of change of pH on the fragility of erythrocytes at constant osmotic pressure has been studied. For this purpose, a special series of isosmotic phosphate buffers had to be prepared. Their preparation is described.

Haemolysis in solutions of a series of alkali halides has shown slight differences between the ten salts studied, but, apart from sodium fluoride, the differences were very small.

It has long been known that lowering the temperature of a system, increases considerably the degree of haemolysis. It has been proved in this work that this is a direct effect of change of temperature on the osmotic pressure of the haemolysing solution in accordance with the laws of physical chemistry. It has been shown that, at constant osmotic pressure, change of temperature has no effect on the degree of haemolysis.

These facts suggest, indirectly, that the erythrocyte is a perfect osmometer, but there is a considerable literature

leading to the conclusion that the red cell is not a perfect osmometer. The evidence for this is due to the use of a faulty method of determining the relative cell volume of swollen erythrocytes by means of the centrifuge. When centrifuging is used, it has been shown that the degree of cell swelling in a hypotonic medium is governed solely by the relative centrifugal force applied. A method has been employed which does not involve high-speed centrifuging of artificially swollen cells and by means of this method, it has been proved that the erythrocyte is indeed a perfect osmometer.

Using the Fiske Osmometer (which is a very accurate instrument for the determination of total osmotic pressure) it has been shown that the intracellular total osmotic pressure of the erythrocyte is the same as the extracellular total osmotic pressure of the plasma in a wide variety of cases, both normal and abnormal.

The total osmotic pressure of each of a series of true body fluids (as distinct from excretions) has been measured by the Fiske Osmometer and compared with the total osmotic pressure of the corresponding serum. The fluids studied included ascitic, cerebrospinal, hydrocele, oedema, pericardial, pleural, spermatocele and synovial fluids. In each case, the fluid was found to have the same total osmotic pressure as that of the corresponding serum within the limits of experimental error. Examination of the electrolyte and protein constituents of these fluids strongly points to the conclusion that neither colloid

osmotic pressure, nor increased capillary permeability, nor membrane damage can be the primary cause of abnormal fluid collections in the human body. The conclusion has been reached that there is a law of constant osmotic pressure of all true body fluids and that this law holds whether the subject is biochemically normal or abnormal.

INTRODUCTION.

The normal human adult has about 3×10^{13} red cells circulating in the vascular system. The average life of the red cell is approximately 120 days so that 2.5×10^{11} cells are destroyed daily and this is equal to a rate of destruction of about 3 million cells per second. The problems of cell regeneration form an immense literature in biochemistry, physiology, and all branches of medicine dealing with the various anaemias, but the problems of cell destruction receive little attention since the haemolytic anaemias form a very small clinical group. In investigating cell destruction, it soon becomes evident that the investigation must be directed to the fundamental problems of the cell rather than to any immediate study of the haemolytic process *in vivo*, and it is to this end that the present manuscript has been compiled.

None of the methods of approach to haemolysis *in vitro* is simple, and all are obscured by our lack of knowledge of the fundamental properties of the erythrocyte and its membrane. The first approach not only shows our ignorance, but reveals the disagreement that exists between different investigators. As an example, it has been argued over the last twenty years and more whether or not the red cell is a perfect osmometer and the balance

of evidence, which is not very convincing, upholds the view that it is not. This is a highly important matter, for if the red cell does not obey the known laws of physical chemistry there must be some reason for it, and presumably it must therefore obey some other laws even to the extent of being a law unto itself. It is an obvious deduction that if the erythrocyte does not obey the laws of physical chemistry in simple test-tube experiments, then any investigation of its behaviour *in vivo* along the lines of classical biological experiment, is a mere waste of time until the basic features of red cell behaviour are known.

Studies of red cell disintegration can be classified into several broad groups:-

(1) Certain crude methods have been used such as the effect of heat, drastic changes in pH, and the action of chemicals. The effects of these agents in producing haemolysis are of little account, but in physiological systems, the effects of change of temperature and pH within biological limits yield interesting information. The study of haemolysis by mechanical shaking is hardly to be classified as "crude" for the red cells are subjected to violent mechanical forces when they are ejected from the left ventricle, and the applied force may have some effect on the continued existence of the older cells in the circulation. But studies of the mechanical destruction of red cells have yielded little

important information.

(2) Lytic agents have been extracted from various organs, especially spleen, muscle and kidney, but it is still debated whether these lysins exist as such and exert their effects in the intact body, or whether they are breakdown products produced chemically or enzymatically in the process of isolation. Whatever the shortcomings of experiments with tissue lysins, it is difficult to avoid postulating a substance or a system which either causes haemolysis or so weakens the cell that it becomes susceptible to the mechanical forces causing disintegration. Final fragmentation is known to occur and the fragmented cells are removed from the circulation by the reticulo-endothelial system.

(3) Osmotic haemolysis is an ideal method of controlled laboratory investigation, and, as will be shown, supplies a goodly part of our fundamental knowledge of cell behaviour. But it is impossible to visualise any conditions in the human body where osmotic pressure could be the controlling factor in producing haemolysis. Studies of osmotic haemolysis are therefore largely academic, but they are closely bound up with the problem of osmotic swelling, which is the stage preceding osmotic haemolysis, and osmotic swelling is of considerable importance.

(4) A considerable literature, due to the investigations of Ponder, has accumulated on the red cell swelling and haemolysis which is brought about

by saponin. The systems used are highly artificial and the only haemolytic compound in blood which is related to saponin is bile acid, and there is no evidence that bile acid plays any important part in normal erythrocyte destruction.

The difficulties associated with *in vivo* studies of haemolysis have more or less forced investigators to study simplified systems in the laboratory. Many of the results cannot even be applied indirectly to investigations *in vivo*, but they do contribute to our knowledge of the red cell. Haemolysis of red cells was first studied by Hamburger about seventy years ago and papers have appeared sporadically since then dealing more with permeability than with actual haemolysis (although the two are very closely linked together). The object of the work described here has been an examination of the factors concerned in *in vitro* haemolysis. The number of known factors is relatively small, and it is quite obvious that any advance in our knowledge of erythrocyte behaviour must depend on the discovery of new facts. It is not proposed at this point to give a complete survey of the literature on haemolysis in general. This has been adequately covered in "Haemolysis and Related Phenomena" by Ponder (1948) and in "The Permeability of Natural Membranes" by Davson and Danielli (1952). Only those fields which have a

direct bearing on the experimental work which follows, will be dealt with.

Attention is first drawn to the impossibility of comparing satisfactorily the results of different publications. In the field of osmotic haemolysis no two authors seem to select the same experimental conditions. Every variety of anticoagulant is used; some prefer venous blood, others arterial (oxygenated) blood; the dilution of blood in the haemolysing system may be anything from 1 in 3 to 1 in 500; some use washed red cells, others use whole blood; the temperature of the system may vary from 4° to 37°C.; and so on. Comparison of results is therefore more likely to result in confusion than clarification. In many cases, the technique employed is open to the gravest suspicion, e.g., those who employ the "drop" as their standard unit of volume, and others who alter the pH by adding hydrochloric acid to red cell suspensions.

In spite of such difficulties, certain points have emerged on which there is general agreement. It has long been known that the degree of haemolysis in hypotonic saline varies inversely with the temperature of the system. There are four major theories (and several minor ones) put forward to explain this phenomenon, but none has ever been supported by any substantial body of experimental evidence. Since the minority of studies on osmotic haemolysis are carried out at body temperature, and

since different observers work at temperatures ranging between 4° and 37°C., this point is one which deserves further attention.

The very fundamental question of whether or not the erythrocyte is a perfect osmometer has already been mentioned and it is quite evident that the compiling of further results by the present methods will merely add to the confusion. If one reads between the lines, it will be seen that the various investigators believe that the erythrocyte is a perfect osmometer but their results show that it is not, and they are rather apologetic about it. Here is a problem in which every detail demands the closest scrutiny.

Experiments in which the rate of permeation of a substance through the cell membrane is measured, must usually be interpreted with caution, especially in the cases of a substance which penetrates rapidly. The rate of haemolysis may be so rapid that the time required for complete haemolysis is less than the time taken to mix the blood with the haemolysing system, so that the rate of haemolysis is largely guesswork. Addition to the system of substances which do not penetrate the cell will increase the extracellular osmotic pressure and will therefore inhibit haemolysis by osmotic forces - apart from any other effects which they may have. For example, if blood be diluted 1 in 20 in isotonic

urea solution, haemolysis is complete almost as soon as the two are mixed; if, however, sodium chloride is also added to give a final concentration of 0.5 g. per 100 ml., no lysis occurs. Frequent references will be made in the text to the difficulties of interpreting experiments on permeability when the system contains appreciable amounts of sodium chloride or other electrolyte, especially sodium oxalate.

There has always been a diversity of opinion on the concentration of sodium chloride which is isotonic (or iso-osmotic) with blood plasma. There is an equal diversity of opinion on the concentration of sodium chloride which is isoplethechontic with blood plasma (Ponder and Saslow introduced the word isoplethechontic, lit. "volume-maintaining", to describe those solutions in which the red cell maintained an unchanged volume). Isotonic sodium chloride is commonly taken as 0.9 g. per 100 ml., but this is no more than a convenient approximation. Values between 0.85 and 1.10 g. per 100 ml. are to be found in the literature. According to Ponder, solutions which are isotonic are not necessarily isoplethechontic because of the doubt which exists about the ability of the red cell to function as a perfect osmometer.

Some writers, Jacobs in particular, attempt to express haemolytic and pre-haemolytic processes in

mathematical terms. While the attempt is praiseworthy, the results are again confusing. The impossibility of determining such quantities as the intracellular pH and the concentration of intracellular non-penetrating neutral molecules, leaves too many unknown quantities in the equations, while the assumptions that only uni-univalent electrolytes need be considered, that all electrolytes have activity coefficients of unity, and so on, raise considerable doubts about the accuracy of the final equations. The truth is that our knowledge of the osmotic behaviour of the red cell is quite insufficient to allow us to express any of its osmotic properties in mathematical terms. It is premature to attempt to express osmotic relationships in the form of equations while the question of the erythrocyte's ability to behave as a perfect osmometer at different external tonicities is still under suspicion. If it is finally proved that the red cell is not a perfect osmometer, all Jacob's equations, and most of Ponder's, become so much waste paper.

The extent to which a cell can swell before the membrane releases the haemoglobin is another point on which opinions clash. According to Ponder (1949-50), the mean cell volume of the human erythrocyte is 86 $m\mu^3$ and the mean cell surface area is 163 $m\mu^2$. A sphere of surface area 163 $m\mu^2$ would have a volume of 193 $m\mu^3$ so that if the erythrocyte swelled to its fullest capacity (without stretching

the membrane) its volume could increase from 86 to 193 $m\mu^3$ i.e., 2.25 times. According to the calculations of Guest and Wing (1942), the cell can swell to only 1.65 times its initial volume without stretching the membrane, but they take the mean cell surface area as 140 $m\mu^2$. All methods of measuring cell surface area are approximations, are mostly based on measurements of the diameter (not a very satisfactory process) and the cell thickness (even more unsatisfactory), and are calculated on the assumption that the cell is a flat cylinder (whereas it is known to be a biconcave disc). Until some new method of measuring surface areas of cells accurately is found, this method of approach will make little progress.

The permeability of the red cell to cations was first established by Davson in 1940 who showed that the cat erythrocyte was demonstrably permeable to both sodium and potassium. Under certain conditions, e.g., when red cells are suspended in hypotonic saline, it has been shown by Davson and others that all erythrocytes (including those of the human) are permeable to both sodium and potassium. The escape of potassium from cells suspended in hypotonic solution was fairly pounced upon as the explanation of the anomalous swelling of the red cell, and of practically every other property of the red cell which could not easily be explained. It was later realised, however, that this escape of

potassium was relatively slow compared with the rate of swelling of the cell, which is exceedingly rapid, and was not the cause of the cell's inability to behave as a perfect osmometer when suspended in hypotonic saline.

These are a few of the problems which face the investigator in this field, and the more one reads in the current journals, the more one is impressed by the necessity of turning to first principles and building up from there by scientific observation. In this branch of physiology, the desire to advance at the expense of forming a solid foundation of facts, is seen at every turn of the page.

The usefulness of measurements of osmotic fragility has been severely criticised by Ponder (1936) in a review dealing with the kinetics of haemolysis. He argues that the fragility of the erythrocyte is affected by four factors, its water content, the osmotic pressure of the interior, the critical volume which it can attain, and the extent to which it behaves as a perfect osmometer. He then concludes,

> "When fragility is measured in the usual way,
> "by putting red cells into hypotonic sodium
> "chloride and observing the concentration
> "(which does not give the true tonicity unless
> "the freezing-point depression of the plasma
> "normally surrounding the cells is known) in
> "which they haemolyse, the result may mean

"anything, and more usually nothing, for such "measurements of fragility amount to measurements "of as many as four variables at one and the "same time."

It may seem unreasonable to quote from a review which was written about twenty-five years ago, but the fact is that no major advances in the problems of osmotic fragility have been reported since, nor have Ponder's views on the subject, as quoted above, been challenged. The obvious reply seems to be that the sooner someone examines these four variables, the better - and it will have been noted that the question again crops up of the "extent to which it (the red cell) behaves as a perfect osmometer". But it requires no specialised knowledge of the field to realise that there are more factors concerned than the four which Ponder has listed. He would have been on safer grounds had he chosen temperature, pH, oxygenation, and the osmotic pressure of the extracellular fluid. And there are several others.

We now turn to brief descriptions of the variables which will be encountered.

(1) Blood Samples. Blood from a great variety of species has been examined, but most of the work recorded in the literature has been carried out with either human or rabbit blood. Many important species differences have been found, e.g., the red cells of the camel are much more resistant to osmotic

haemolysis than those of any other species; they do not haemolyse completely until the extracellular sodium chloride concentration is reduced to about 0.28 g. per 100 ml. In this work, human blood has been used throughout; in a few experiments, human umbilical cord blood has been used.

(2) Age and Sex. The age and sex of the blood donor seem to be immaterial.

(3) Anticoagulants. Oxalate and heparin are most commonly used but defibrinated blood has also been employed, although not in this work. The objection to the use of oxalate is that a very large excess in anticoagulant is generally used, and this excess remains in the plasma - increasing its tonicity. A slight excess of oxalate is of no consequence if dilute systems of blood are used, e.g., one volume of blood added to 20 or more volumes of hypotonic saline, nor is it of any consequence if washed cells are used. Heparin is the better anticoagulant. Defibrination may cause mechanical damage to the cells.

(4) Use of Whole Blood or Washed Cells. Both systems have been used extensively. The advantage of washed cells is the absence of any "plasma effect", but for accuracy of manipulation the cells must be suspended in a small volume of isotonic saline and this has its own complications. The possibility of mechanical damage to the cells during the washing is small, but repeated washing and centrifuging delays

the start of experiments, and the permeability and fragility of the cells change on standing. It is said that washed cells may be stored at refrigerator temperature without alteration of their osmotic properties, but there is a good deal of evidence against this, e.g., the escape of potassium which occurs when cells stand in saline solution for many hours. The age of the blood specimens is a point to consider.

(5) Oxygenation. Most experiments recorded in the literature have been carried out using either venous blood or the washed cells from venous blood. Jacobs and Parpart (1931) were the first to show that oxygenation decreased the fragility of red cells. Other authors have reported the difference in fragility of the cells of venous, capillary, and arterial (oxygenated) bloods. The difference is considerable and has been further investigated in the present work.

(6) The Haemolysing System. The great majority of studies of osmotic haemolysis have employed dilute solutions of sodium chloride as the haemolysing system. In a small minority of cases, the haemolysing system has been a dilute solution of other alkali halides, diluted plasma or serum, or dilute solutions of non-electrolytes. In a few cases, complex mixtures of salts (sodium, potassium, calcium and magnesium, chloride, bicarbonate, phosphate and oxalates) have been employed chiefly

with the object of controlling the pH. The great advantage of a simple solution such as sodium chloride solution, is the ease with which its osmotic coefficient may be calculated, but biologically, diluted plasma should be the fluid of choice.

(7) Dilution of the Blood. When whole blood is used, the ratio in which it is mixed with a hypotonic haemolysing system directly affects the degree of haemolysis and cell swelling. The greater the proportion of blood in the system, the greater the amount of added plasma and the greater, therefore, the osmotic pressure of the extracellular fluid, and vice versa. Small variations in the dilution do not greatly affect the degree of haemolysis, and the dilution selected is not a matter of fundamental importance, provided that it is kept constant in any series of experiments. In this work, a dilution of 1 part of blood in 20 parts of haemolysing system has been chosen largely as a matter of convenience.

(8) Time Factors. There are two important time factors involved in osmotic haemolysis.

(a) The time interval between the withdrawal of the blood and its distribution into the haemolysing system. The effect of this time interval is, as will be shown, closely bound up with the process of oxygenation. The fragility of venous blood decreases if the blood is allowed to stand with occasional mixing, and

this may be a serious source of error in the accurate determination of fragility.

(b) The second time factor is the length of time that the blood is allowed to remain in contact with the haemolysing system. Osmotic haemolysis is a very rapid process. The great majority of cells which are destined to haemolyse under a given set of conditions, do so in a few seconds - hence the great difficulty of devising accurate methods of measuring the rate of osmotic haemolysis. In any partially haemolysed system, there must be a number of cells on the verge of (irreversible) lysis, so that the degree of haemolysis will increase very slowly with time.

(9) Hydrogen Ion Concentration. In all biological work, the pH must be under control and the study of haemolysis is no exception. It will be shown later that the degree of haemolysis varies markedly with change in pH, and herein lies one of the main reasons for using whole blood in preference to washed cells, for the added plasma, although it may complicate matters, goes a long way toward stabilising the pH, and change in hydrogen ion concentration is undoubtedly the cause of many abnormal and unexpected results.

Simmel (1923) introduced a buffered haemolysing system containing, amongst other things, sodium dihydrogen phosphate and sodium bicarbonate. The

bulk of the osmotic pressure of Simmel's solution, however, was still due to sodium chloride so that the buffering power was strictly limited. Nevertheless it was sound in principle for it aimed at controlling an important variable. Simmel's solution was never popular, solutions of plain sodium chloride being preferred for clinical work, but it is interesting to note the introduction in the last year or so of a standard solution for cell fragility work containing sodium chloride, sodium dihydrogen phosphate and disodium hydrogen phosphate.

Cases are on record where the pH of red cell suspensions has been adjusted by the addition of dilute hydrochloric acid.

(10) Temperature. It has long been known that the degree of haemolysis alters with change in temperature - the higher the temperature, the less the degree of haemolysis, and vice versa. The temperature coefficient of osmotic haemolysis is not very great and temperature control to within 1°C. is sufficient to eliminate this variable. Ideally, experiments should be conducted at 37°C. which would entail the use of apparatus warmed to this temperature. The technical difficulties would be considerable.

The cause of this change in osmotic fragility with change of temperature has been extensively studied in this work. Temperature fluctuations in any individual experiment must be avoided.

(11) Measurement of Haemolysis. The earliest quantitative method of measuring the degree of haemolysis consisted of counting the unhaemolysed cells in a haemocytometer. This tedious and inaccurate method is now replaced by the determination of the percentage of the total haemoglobin which has been liberated into the cell-free supernatant. This latter method is both accurate and rapid. It is based on the assumption that haemolysis obeys the all-or-none law - a point which is examined later.

(12) The Erythrocytes. The degree of haemolysis depends on the osmotic resistance of the average cell in the sample. With normal blood, the degree of haemolysis is remarkably constant provided that the various factors which have been mentioned above are kept constant. Variations of any significance are practically confined to the increased fragility of the cells in certain cases of haemolytic anaemia, and the decreased fragility found in cases of jaundice.

(13) Erythrocyte Swelling. Cell swelling and haemolysis are closely related, the latter being the end point of the former so that one may work in terms either of the degree of haemolysis or of the extent of cell swelling prior to haemolysis. The important point to stress at this stage is that, while different information may be gathered by these two methods of approach, the results should be

in agreement in fundamental matters.

Cell swelling is closely related to the conversion of the red cell from a biconcave disc to a sphere, and the study of the action of sphering agents, including hypotonic saline, is interesting. Whether or not these sphering agents are capable of bringing about the disc-sphere transformation without change in volume, is a fine point. Ponder claims that they can.

When all possible variables are considered it is easy to understand the difficulties of establishing standards and comparing the results of different workers. Every laboratory has established its own standards. But there is more to the matter than this, for many conclusions require to be re-examined and much remains to be discovered. It is the writer's view that the problems must be tackled by the methods of chemistry and biochemistry, and that it is futile to attempt a mathematical analysis before the elementary facts have been established. Having established the facts, theoretical deductions may or may not be possible. These rather obvious points require to be emphasised, and attention may again be drawn to the tendency in work of this type to make unwarranted assumptions and to accept facts on the basis of experimental work which is open to grave suspicion.

In essence, the first part of the work reported in this thesis is an examination of the fundamental properties of the red cell. Certain of the factors mentioned above have been examined in detail - the effect of oxygenation, of change of pH and temperature, of using improved methods of technique, etc., all leading up to the most important point of all - the ability of the normal erythrocyte to act as a perfect osmometer.

The osmotic properties of the erythrocyte are inextricably bound up with the osmotic properties of the plasma in which it is suspended. The osmotic pressure of plasma has received remarkably little attention for the simple reason that observations on the red cell were so much easier to make - especially if one were observing haemolytic phenomena. At least two methods of investigating the osmotic pressure of extracellular fluids have long been available: the vapour pressure method of Hill (1930), and the cryoscopic method based on the Beckmann thermometer. The former involves some elaborate apparatus and is time-consuming, while the latter is tedious to a degree and the super-cooling, which cannot be avoided with biological fluids, makes the results a little more than suspect. And this aspect of the problem is every bit as important as the osmotic properties of the cell.

All the difficulties of determining osmotic

pressure were resolved a few years ago by the introduction of the Fiske Osmometer which is essentially an instrument for the rapid determination of the freezing points of solutions. It is a most ingenious piece of apparatus which entirely eliminates all errors of supercooling, and it is so constructed that a series of readings of the osmolarity of any given solution can be made at the rate of one every three minutes or so. It is an instrument which can be properly appreciated only by those who have served a long apprenticeship with the conventional Beckmann thermometer method. Its appearance in this country in 1959 has made possible extensive investigations of the osmotic pressure of body fluids and the last part of this work deals with this subject. The existing literature on the total osmotic pressure of human body fluids is very scanty.

Serum is the most readily available of all body fluids and is generally accepted as representative, in terms of total osmotic pressure, of all the body fluid compartments. This is based on the assumption that there are no barriers to the movement of water between these compartments, and that movement of water alone can maintain the total osmotic pressure on both sides of any membrane. Although perhaps not strictly accurate, this statement can be accepted as a first approximation. The difference in total osmotic pressure between serum and plasma

must be very small; the only major chemical difference is the occurrence in native plasma of a low concentration of fibrinogen (mol. wt. 400,000) which cannot appreciably affect the total osmotic pressure.

The osmotic pressure of normal serum has been determined by Culbert (1935), Benham, Duke-Elder and Hodgson (1938), Lifson (1944), and by Olmstead and Roth (1957). Their results are all in good agreement. Apart from these, an examination of the literature of the past 30 years has disclosed only two substantial accounts dealing with the osmotic pressure of other body fluids.

The first of these is a paper by Makepeace, Fremont-Smith, Dailey and Carroll (1931) which gives many simultaneous determinations of the freezing points of serum and amniotic fluid. In spite of certain technical difficulties, including an occasional considerable lapse of time between the collection of the two fluids (making comparison difficult), and all the troubles inherent in the Beckmann thermometer method, their results have established that the amniotic fluid at term is significantly hypotonic to the corresponding serum, and it is unlikely that any of the technical difficulties could account for the extent of the difference. This, and certain chemical differences, led these authors to conclude that the amniotic

fluid at term is diluted with foetal urine - which latter is known to be decidedly hypotonic to normal serum. Their conclusions are now generally accepted and amniotic fluid at term (being admixed with a secretion) is not regarded as a true body fluid in the sense used here. The second report is by Blegen (1939) on the total osmotic pressures of simultaneously collected specimens of serum and cerebrospinal fluid determined by the vapour pressure method of Hill. In 27 of the 29 cases he studied, the total osmotic pressure of the serum was greater than that of the corresponding cerebrospinal fluid, but the average difference was less than 1% and is probably within the experimental limits of the method.

A similar type of problem is the relationship between the total osmotic pressures of intracellular and extracellular fluids. Earlier work had appeared to show most emphatically that the osmotic pressure of tissues was very much greater than that of the extracellular fluid, but a great deal of convincing evidence has lately been produced to prove that this difference was an artefact due to autolytic change and that the two osmotic pressures were the same (Conway and McCormack (1953), Appelboom (1957), Maffly and Leaf (1958), and others). The whole matter has been discussed at length by Conway (1957) and the conclusion was reached that the total osmotic pressure of the two fluids

(intracellular and extracellular) were identical.

The main object of this part of the work, however, was to compare the total osmotic pressure of true body fluids (as distinct from excretions such as urine, bile, saliva, etc.) with that of the corresponding serum. No attempt has been made to accumulate data for the purposes of establishing means and standard deviations. Only two of the body fluids are obtainable in sufficient quantity from normal subjects. Venepuncture is an innocuous process, but lumbar puncture is not to be recommended except as a clinical necessity. The other fluids (ascitic, pleural, and so on) accumulate in quantity only in abnormal cases, and since the abnormalities in such cases are the results of physical and chemical aberrations, it is necessary to compare the total osmotic pressure of each fluid with that of the corresponding serum, which latter functions as a "standard". Some of these fluids, and the sera, were biochemically abnormal, but this merely serves to emphasise the points which will be made.

Part 1.

Determination of the Degree of Haemolysis.

Human blood has been used in all the following work. In most cases, it was obtained from normal, healthy blood donors attending the Scottish National Blood Transfusion Service. The anticoagulant used was either heparin or oxalate. In the case of oxalate, a standard solution was prepared according to the directions of Heller and Paul (1934) containing 2.0 g. potassium oxalate and 3.0 g. ammonium oxalate per 100 ml. 0.04 ml. of this solution (evaporated to dryness in an electric oven at 120°C.) was used to prevent the coagulation of each ml. of blood withdrawn. With heparin, 1 drop of B.D.H. heparin solution (evaporated to dryness in a vacuum desiccator), or 1 mg. of heparin powder, was used to prevent the coagulation of 5 - 10 ml. of blood. In any series of experiments, the same anticoagulant was used throughout. All experiments, unless otherwise stated, were started within 30 minutes of the blood having been withdrawn. Glassware was cleaned by dichromate-sulphuric acid mixture, washed well, allowed to soak for several hours in tap water, and finally washed with distilled water before drying. All burettes and pipettes were calibrated.

In determining the degree of haemolysis, the blood was diluted 1 in 20 in the haemolysing system by adding 0.500 ml. blood to 9.50 ml. of the solution.

The tube was closed with a clean, dry stopper, the contents mixed by inversion, and then allowed to stand for a definite period (a few minutes to several hours in different experiments) at constant temperature. The contents were again mixed and the tube centrifuged at about 2,500 r.p.m. for 2 - 3 minutes. The clear supernatant was removed and its haemoglobin content determined.

In each experiment, one tube to which 0.500 ml. of blood was added, contained 9.50 ml. of "normal saline" and it was treated in the same way. The reading obtained constituted the "blank". Haemolysis never occurred in this tube but the "blank" always showed some slight degree of light absorption due to (a) plasma proteins and lipoids, and (b) the plasma pigments. This "blank" was used to correct all other readings in the same experiment. The correction was very small; it never reached the equivalent of 1% haemolysis.

In each experiment also, one tube to which 0.500 ml. of blood was added, contained 9.50 ml. of distilled water, and this tube was treated in the same way. In distilled water at this dilution, haemolysis is rapid and complete, and the liberated haemoglobin (corrected for any blank) represents 100% haemolysis.

The haemoglobin in the supernatant fluid was determined by the alkaline haematin method of Clegg and King (1942). 2.00 ml. of supernatant was

transferred to a tube graduated at 10.00 ml. Approximately 8 ml. of N/10 sodium hydroxide was added and the mixture allowed to stand 5 - 10 minutes at room temperature. The contents were made up to 10.00 ml. with alkali, mixed, and the colour density measured in a Hilger Photoelectric Absorptiometer using green filters with distilled water as the standard. It is not necessary, as in Clegg and King's method, to heat the tubes at 100° for 4 minutes unless one is dealing with foetal blood the haemoglobin of which is resistant to N/10 alkali at room temperature. With adult blood, heated and unheated specimens have exactly the same extinction coefficient. If the haemolysing solution contains much carbohydrate, heating with N/10 alkali produces caramelisation with production of a yellow colour in the blank.

If, A = the reading of the blood-haemolysing solution mixture,

B = the reading of the blood-saline mixture, and

C = the reading of the blood-distilled water mixture, then,

$$\frac{(A - B) \times 100}{(C - B)} \%$$

is the percentage haemolysis.

This formula depends on the degree of light absorption being directly proportional to the concentration of alkaline haematin over the range of

concentrations encountered in these experiments. It is found that this is so. Crystalline haemin (Fe = 8.48%; calculated for $C_{34}H_{32}O_4N_4FeCl$, Fe = 8.57%) was prepared by the method of Delory (1943) and it was found that both haemin and blood in N/10 sodium hydroxide obey Beer's Law over a wide range of concentrations.

The figures for the percentage haemolysis are independent of the haemoglobin and the red cell count of the blood specimen, except insofar as a diminished cell count implies an increased percentage of plasma in the added blood, and this in turn will increase the osmotic pressure of the haemolysing system. The packed cell volume of normal blood is reasonably constant and corrections for the added plasma need be applied only when dealing with cases of marked anaemia.

When blood is haemolysed completely in distilled water and the mixture centrifuged, a small amount of deposit invariably settles out. This is a mixture of γ-globulins and ghost cells. The deposit, on washing with distilled water, is usually pure white, but may occasionally be pink. The pink colour, when present, cannot be removed by repeated washing with water or with saline, and is presumably due to the presence of a minute amount of haemoglobin adhering to the stromatin of the cell. Why this should occur with some specimens of blood and not

with others, is not known. A coloured deposit, no matter how often it be washed, always gives a strong benzidine reaction, but it never contains any structure which can be identified microscopically as an intact erythrocyte. Attempts to determine the amount of haemoglobin remaining attached to the stroma from 0.500 ml. of blood have not been successful. The amount is exceedingly small and would correspond to less than 0.04% haemolysis in any tube.

In no experiment has there been any indication that, at a dilution of 1 in 20, anything short of complete haemolysis occurred in distilled water. It is unnecessary to add ammonia, saponin, or any other haemolytic agent to ensure complete haemolysis.

Part 2.

Factors concerned in Osmotic Haemolysis.

The "All-or-None" Law in Haemolysis.

It is of primary importance to determine whether "50% haemolysis" means that 50% of the total cells have given up the whole of their haemoglobin, or whether each cell has given up 50% of its haemoglobin (an unlikely event), or whether all cells have parted with varying amounts of their haemoglobin giving an apparent value of 50% haemolysis.

The problem is solved in the following way. 0.500 ml. of normal blood is added to 9.50 ml. of a series of solutions of sodium chloride of varying concentration - each giving a different degree of haemolysis. After standing for 2 hours, the mixtures are centrifuged and as much of the supernatant as possible removed by a dry pipette, care being taken to disturb none of the sedimented cells. The percentage haemolysis is determined on this supernatant. The supernatant is immediately replaced by an equal volume of 0.90% sodium chloride solution, which stops all tendency to further haemolysis. The unhaemolysed cells are gently mixed with the saline, the mixture is centrifuged, and the supernatant discarded. The washing is repeated three times in all to remove the last trace of extracellular haemoglobin. The washed cells are transferred quantitatively to a graduated tube and are finally suspended in 10.00 ml. saline. In this

suspension, the total haemoglobin and the total red cell count are determined.

Table 1. The Mean Cell Haemoglobin and Colour Index of the Unhaemolysed Cells at varying Degrees of Haemolysis. (Adult Blood).

	Blood Specimen No. 1.				
NaCl Concentration. g. per 100 ml.	0.900	0.425	0.410	0.395	
Percentage Haemolysis.	0	6	15	44	
Total Haemoglobin (mg.)	74.5	68.5	62.5	39.2	
Total Cells (x 10^9).	2.72	2.26	2.14	1.19	
M.C.H. ($\mu\mu$g.).	27.4	30.3	29.2	33.0	
Colour Index.	0.93	1.03	0.99	1.12	
	Blood Specimen No. 2.				
NaCl Concentration. g. per 100 ml.	0.900	0.425	0.410	0.395	0.380
Percentage Haemolysis.	0	23	46	70	82
Total Haemoglobin (mg.)	77.5	58.5	37.7	23.1	12.6
Total Cells (x 10^9).	2.37	1.90	1.08	0.74	0.41
M.C.H. ($\mu\mu$g.).	32.7	30.8	34.8	31.3	30.5
Colour Index.	1.11	1.04	1.18	1.06	1.03

From the figures obtained, the mean corpuscular haemoglobin is calculated, or, taking the normal mean corpuscular haemoglobin as 29.5 micro-micro-grams, the colour index of the unhaemolysed cells in each tube is calculated. The results obtained with two different specimens of adult blood are shown in

Table 1.

In another series of experiments, umbilical cord blood was used in place of adult blood. The foetal cells also obey the all-or-none law.

Table 2. The Mean Cell Haemoglobin and Colour Index of the Unhaemolysed Cells at varying Degrees of Haemolysis. (Umbilical cord blood).

	Blood Specimen No. 1.			
NaCl Concentration. g. per 100 ml.	0.480	0.440	0.400	0.360
Percentage Haemolysis.	5	23	48	72
Total Haemoglobin (mg.).	86.0	69.6	42.5	25.3
Total Cells (x 10^9).	2.34	1.97	1.12	0.698
M.C.H. ($\mu\mu$g.).	36.8	35.4	38.0	36.3
Colour Index.	1.25	1.20	1.29	1.23
	Blood Specimen No. 2.			
NaCl Concentration. g. per 100 ml.	0.440	0.400	0.360	0.320
Percentage Haemolysis.	13	43	70	83
Total Haemoglobin (mg.)	65.8	41.1	17.8	6.2
Total Cells (x 10^9).	1.98	1.18	0.570	0.154
M.C.H. ($\mu\mu$g.).	33.2	34.8	31.2	40.4
Colour Index.	1.13	1.18	1.06	1.37

Making allowance for technical difficulties in the accurate counting of the red cells and for the fact that the mean corpuscular haemoglobin (and the colour index) are derived from two experimental

figures, it is clear from the data in these Tables that the mean cell haemoglobin of the unhaemolysed cells remains constant at different degrees of haemolysis. If each cell liberated only part of its haemoglobin, a progressive fall in the mean cell haemoglobin would occur, and the lowest figure for this index would be found in the tubes with the most extensive haemolysis. Since such a fall does not occur, one may safely assume that the "all-or-none" law is followed in haemolysis. There is some critical point at which the individual cell decides to haemolyse and it then loses the whole of its haemoglobin; and this critical point occurs at different salt concentrations for different cells.

It is of interest to note in passing, that the presence of "ghost" cells does not interfere with the haemocytometer counts unless one is dealing with a sample which has been haemolysed to the extent of 80% or more. Up to this degree of haemolysis, ghost cells are very seldom seen, but beyond this point, they make cell-counting difficult but not inaccurate.

Oxygenation and Osmotic Fragility.

Time Factors in Haemolysis Experiments.

A few preliminary experiments carried out at an early stage made it very evident that the lengths of time which elapsed between the several stages of an experiment made a considerable difference to the

final degree of haemolysis. There are two important "time factors" in each experiment, and these are:-

(a) the length of time elapsing between the withdrawal of blood from a vein and its distribution into the haemolysing solution, and, (b) the length of time which the cells remain in contact with the haemolysing solution.

Since the second of these is the simpler, it is more convenient to discuss it first.

Partial haemolysis in hypo-osmotic saline is not an instantaneous phenomenon. The majority of the cells which are destined to haemolyse do so as soon as mixing is complete (a matter of a few seconds), but the process does not stop at that point. In any system where partial haemolysis has occurred there must always be many cells hovering on the verge of haemolysis, and since the process is irreversible, a very gradual increase in the degree of haemolysis is to be expected. The cell membrane is not completely impermeable even to cations when it is stretched to near its limit as it is immediately prior to haemolysis. This gradual increase may be shown by adding 0.500 ml. blood to each of several tubes containing 9.50 ml. of 0.410% sodium chloride and allowing the tubes to stand for varying periods before centrifuging. The following Table (Table 3) shows the gradual increase in the degree of haemolysis which occurs when either venous or oxygenated blood is used.

The last three samples were fully oxygenated by exposure of the blood to an atmosphere of pure, moist oxygen in a tonometer for 15 - 20 minutes, with mixing at frequent intervals, prior to distribution into the saline solutions.

Table 3. Showing the Increase in the Degree of Haemolysis when Erythrocytes stand in Contact with Sodium Chloride Solution. Concentration = 0.410 g. NaCl per 100 ml. Dilution = 1 in 20. Temperature = 18.5°C. The blood was obtained from 5 different individuals.

	Percentage Haemolysis.						
Time in hours.	½	1	2	3	4	5	8
1. Venous	35	-	39	40	47	-	-
2. Venous	48	-	49	51	-	56	57
3. Oxygenated	14	-	15	14	-	16	18
4. Oxygenated	20	23	-	27	28	31	-
5. Oxygenated	23	27	28	28	30	29	-

There is a slow rise in the degree of haemolysis the longer the cells are allowed to stand in contact with the saline, and, contrary to the findings of Creed (1938), this increase occurs with both venous and oxygenated blood. The increase, however, is very small.

By similar experiments, it can be shown that this increase does not occur in a solution of 0.90% sodium chloride where there is no haemolysis, nor in distilled water where haemolysis is complete, but

only in those concentrations of sodium chloride which give rise to a partial haemolysis.

The factors governing this increase are not known but they must be concerned with an alteration in the permeability of the cell membrane. For the present purpose, it is sufficient to note that this variable is small and can be eliminated by allowing the cell suspension to stand for a fixed period before centrifuging.

The other time factor (the time elapsing between the withdrawal of the blood and its distribution into saline) is more interesting and much more important. Its study has led, by a rather roundabout route, to a phenomenon already described in the literature. Stated briefly, the longer the blood is allowed to stand before being distributed into the haemolysing solution, the smaller is the observed degree of haemolysis. In the experiments shown in Table 4, 10 ml. of blood was withdrawn and placed in an oxalated tube of about 16 ml. capacity. At the stated intervals, the blood was thoroughly mixed, and 0.500 ml. was transferred to 9.50 ml. of 0.410% sodium chloride solution. The cells were left in contact with the saline for two hours before centrifuging and determining the degree of haemolysis. The temperature was constant at 20°C., and blood from two different individuals was used.

When the percentage haemolysis is plotted against time, both sets of figures give smooth parabolic

curves. The marked decrease in haemolysis cannot be due to bacterial contamination which would act in the opposite direction. Since the rate of change is most rapid immediately after the blood is withdrawn, this variable requires close attention. And the change in the degree of haemolysis is considerable.

Table 4. Showing the Decrease in the Degree of Haemolysis when Venous Blood is allowed to stand before Distribution into Saline. Dilution = 1 in 20.

	Percentage Haemolysis.	
Time Interval	Blood A.	Blood B.
2 minutes	75	87
15 minutes	64	84
30 minutes	57	80
45 minutes	55	-
1 hour	49	75
2 hours	43	71
5 hours	36	65
9 hours	30	57

Whitby and Hynes (1935) have already commented on the difference in fragility between the erythrocytes of venous and arterial (i.e. oxygenated) blood. The cells of the latter are significantly more resistant to osmotic haemolysis, and these writers conclude that the difference is due to the more perfect oxygenation of the arterial blood.

In the two experiments described above it was

observed that, with the frequent mixing of the blood and the opening of the tubes at intervals, the blood became more visibly oxygenated, and it was concluded that the changes in fragility in these two experiments, and the changes in fragility on oxygenation, were one and the same thing. This conclusion is confirmed by a comparison of the fragility of venous and oxygenated blood, and by experiments similar to those described above with the single difference that the blood was fully oxygenated immediately after its withdrawal and prior to the start of the experiment proper.

Table 5. Showing the Absence of Decrease in the Degree of Haemolysis when Oxygenated Blood is allowed to stand before Distribution into Saline.

	Percentage Haemolysis.			
Time Interval.	Blood A	Blood B	Blood C	Blood D
30 minutes.	21	61	36	71
2 hours	24	62	34	72
4 hours	25	60	36	72
5 hours	25	62	35	70
8 hours	25	61	39	73

The pronounced drop which occurs with venous blood is abolished by oxygenation. The process of oxygenation evidently stabilises the blood in a very satisfactory manner as far as this "time factor" is concerned.

The Oxygen Content of Blood and its Fragility.

The relation between the oxygen content of the blood and the osmotic resistance of the cells is readily shown by keeping part of a sample of blood in the venous state, and oxygenating the other part. Both venous and oxygenated bloods are then distributed in the same concentration of hypo-osmotic sodium chloride solution, and the percentage haemolysis determined in both cases. The differences are shown in Table 6 (infra). The drop in the percentage haemolysis after oxygenation is considerable and would obviously have a profound effect on any haemolysis curve, and indeed, on experiments of any kind in osmotic haemolysis. Why the simple process of oxygenation (i.e. conversion of reduced haemoglobin to oxyhaemoglobin) should produce such a striking alteration in fragility has never been explained and deserves further investigation.

Venous blood from a healthy subject still has about 65% of its haemoglobin in the form of oxyhaemoglobin, and the rest in the form of reduced haemoglobin. Since oxygenation of this reduced haemoglobin produces a dramatic increase in the resistance of the cells to saline solution, one would expect that the removal of the oxygen from the remaining 65% of oxyhaemoglobin would bring about an even more dramatic decrease in the osmotic resistance of the cells. This, however, is not the case.

In the next series of experiments, a sample of normal oxalated blood was divided into three parts. The first part was kept in the venous state: the second was fully oxygenated: and the third was de-oxygenated. De-oxygenation was effected by exposing the blood to a vacuum of about 15 mm. Hg until no further evolution of gases occurred. This exposure should not be unduly prolonged as there is some danger of producing a haemoconcentration by evaporation. Rapid production of a vacuum, on the other hand, results in an awkward frothing, but with careful manipulation, the bulk of the oxygen can be removed in about 15 minutes. In this series, blood from 7 different individuals was used.

Table 6. The Effect of Oxygenation and De-oxygenation on the Fragility of Normal Erythrocytes. Temperature, Dilution, and Time of Haemolysis were constant.

	Percentage Haemolysis.		
Concentration of NaCl (g. per 100 ml.)	Venous.	Oxygenated.	De-oxygenated
1. 0.410	20	8	7
2. 0.410	32	10	12
3. 0.410	22	6	6
4. 0.395	61	22	23
5. 0.395	47	7	8
6. 0.380	67	11	10
7. 0.380	71	17	16

Oxygenation and de-oxygenation have the same

effect - a marked increase in the osmotic resistance of the cells. And furthermore, the magnitude of the changes on oxygenation or de-oxygenation are almost exactly the same in all seven experiments.

These figures leave no doubt whatever that the alteration in fragility on oxygenation has nothing to do with the uptake of oxygen or the conversion of reduced haemoglobin to oxyhaemoglobin.

Oxygenation and de-oxygenation in vacuo have one thing in common; both processes will remove other gases in solution or in loose combination with the constituents of the blood. The only gases that can be involved are carbon dioxide, nitrogen, and (possibly) carbon monoxide in the blood of smokers. The last of these can be ruled out since carboxy-haemoglobin is such a stable compound that it is dissociated only very slowly by exposure of blood to an atmosphere of oxygen or to a vacuum. Nitrogen is so classically inert that it is most improbable that its presence or absence has any effect on the osmotic resistance of cells. Attention must therefore be directed to exchanges in carbon dioxide.

Since the blood for these experiments was not drawn off under oil, its pH must be slightly higher than that of venous blood in vivo, due to slight losses of carbon dioxide during the process of withdrawal and the subsequent manipulations. There are many technical objections to the use of paraffin oil during venepuncture and in this type of

experiment comparative figures of value can be obtained without its use. A few preliminary experiments showed that when venous blood was exposed to an atmosphere of oxygen or to a vacuum, there was a change in pH of the order of 0.4 unit, and the change was in the same direction (an increase) in both cases. In one such experiment, the following figures were obtained:-

Venous blood	pH = 7.59
Oxygenated blood	pH = 7.96
De-oxygenated blood	pH = 8.09

These changes in blood pH do not themselves cause haemolysis of the whole blood; they exert their influence only after the blood has been added to a hypo-osmotic solution which will cause partial haemolysis, and it is therefore the pH of the suspension of the red cells in the haemolysing system which is the controlling factor.

When these three samples of blood were diluted 1 in 20 in 0.395% sodium chloride solution, the pH values of the mixtures were:-

Venous blood	pH = 7.60
Oxygenated blood	pH = 7.85
De-oxygenated blood	pH = 7.86

The pH changes are now smaller but they show that the buffers of blood are unable to maintain a constant pH when blood is exposed to an atmosphere of oxygen or to a vacuum.

It is not justifiable at this stage to conclude

that oxygenation (or de-oxygenation) produce their effects on cell fragility by causing an alteration in pH. But oxygenation (or de-oxygenation), and an increase in pH cause the fragility to alter in the same direction, and it is likely that they are interconnected. The next step is to examine the fragility of erythrocytes under conditions of controlled pH.

The Relationship between Fragility and pH.

The use of a buffered haemolysing system in place of sodium chloride solution would substantially reduce, or eliminate entirely, the effect of a change of pH consequent on the removal of carbon dioxide (whether by oxygenation or by de-oxygenation), provided that such effects are reversible; for removal of the carbon dioxide alters the pH of the whole blood, while the influence of the buffering power of a haemolytic system will not be manifest until after the blood is mixed with it.

Theoretical considerations show that no one stable inorganic salt exists which is capable of acting as a buffer against acid and alkali in the pH range 7.2 - 7.8. Any salt derived from a weak acid and a weak base is too unstable, and rather than revert to amphoteric organic compounds (e.g. glycine) it was decided to experiment with mixtures of inorganic salts. Simmel's solution (vide infra) is quite unsuitable since almost all the osmotic pressure is contributed by salts which have no

buffering power. The ideal haemolysing system for the present purpose is one which has all its osmotic pressure derived from salts which are capable of acting as buffers, so that the greatest possible buffering capacity is present in any given hypo-osmotic solution. A system conforming to these requirements is the mixture of phosphates commonly employed, viz, disodium hydrogen phosphate and potassium dihydrogen phosphate.

Although there are certain objections (which will be discussed fully in a later section) to the use of such a buffer, it has served its purpose admirably in these preliminary experiments. Solutions of disodium hydrogen phosphate were made up from a recrystallised sample of the dodecahydrate; a good sample of this salt is essential as it is markedly efflorescent. Potassium dihydrogen phosphate crystallises in the anhydrous state. The haemolysing system employed was a buffer made by mixing equi-molar solutions of these salts so that the pH of the system varied while the total molarity remained constant. The commonly employed M/15 solutions have an osmotic pressure so high that no haemolysis occurs; the molarity was therefore reduced to M/28.5.

Although the total molarity is constant, the osmotic pressures of different mixtures of these solutions, is not - it depends on the ratio of the

two salts. Equi-molar solutions of these two salts are not iso-osmotic. This however, does not affect the present arguments since the object of these experiments is to compare the behaviour of venous and oxygenated bloods, and nothing more. It does become an important factor when a mixture of phosphates is used as a haemolysing system in the determination of fragility curves.

In the following experiments (Table 7), 0.500 ml. of venous or oxygenated blood was added to 9.50 ml. of M/28.5 buffer solution and the mixture was allowed to stand for 2 hours at 19°C. Blood from three different individuals was used. pH determinations were made with a Marconi glass-electrode pH-meter.

Table 7. The Effect of Oxygenation on the Fragility of Erythrocytes in presence of a Phosphate Buffer.

	pH of the original buffer.	Percentage Haemolysis	pH of the Blood-buffer mixture.
Venous blood	7.32	80	7.30
Oxygenated blood		83	7.33
Venous blood	7.51	56	7.49
Oxygenated blood		58	7.50
Venous blood	7.70	33	7.62
Oxygenated blood		37	7.68

At first sight, it appears that oxygenation under conditions of controlled pH, increases slightly the degree of haemolysis. It has not been possible in these experiments, with the low osmotic

pressure necessary to produce a reasonable degree of haemolysis, to have buffers sufficiently concentrated to prevent slight changes in pH. The pH of the mixtures containing oxygenated blood (column 3) is always slightly higher than that of the corresponding system containing venous blood, presumably due to the loss of carbon dioxide during oxygenation.

This much may be concluded - that control of the pH eliminates the fall in the degree of haemolysis which occurs on oxygenation, and this is further evidence that oxygenation *per se* does not affect erythrocyte fragility directly, but affects it indirectly by raising the pH.

The Role of Carbonic Anhydrase.

The carbon dioxide of blood (cells and plasma) is largely in the form of bicarbonate. The bicarbonate ion breaks up *in vitro* and *in vivo* according to the equation:

$$HCO_3^- = OH^- + CO_2.$$

The carbon dioxide escapes into the gaseous phase (test-tube or alveoli) leaving the hydroxyl ion to raise the pH. Meldrum and Roughton (1932) showed that, in blood, the liberation of carbon dioxide from bicarbonate is catalysed by carbonic anhydrase. This enzyme is present in cells but not in plasma.

If the changes in the degree of haemolysis following oxygenation are, in fact, due to loss of carbon dioxide and a consequent rise in pH, one

would expect them to be directly connected with the activity of carbonic anhydrase. Like all enzymes, carbonic anhydrase is sensitive to certain poisons, and Keilin and Mann (1941) have shown that it is especially sensitive to the presence of potassium cyanide and to sulphanilamide.

Experiments using potassium cyanide have been unsatisfactory. This is a salt which undergoes extensive salt hydrolysis in solution, and its addition to any haemolysing system produces large changes in the pH - the very thing to be avoided here. In addition, haemolysis is brought about by such low concentrations of cyanide that this ion appears to have some specific lytic action.

Sulphanilamide is preferable. Its presence may have any combination of three possible effects:-

(a) inhibition of carbonic anhydrase activity,

(b) a specific haemolytic (or anti-haemolytic) activity, and,

(c) an increase in the osmotic pressure of the haemolysing system. This, of course, is unavoidable.

The following experiments were designed with the object of showing that inhibition of carbonic anhydrase activity by sulphanilamide prevents the fall in the degree of haemolysis which occurs on oxygenation, and that this compound has no specific haemolytic (or anti-haemolytic) activity.

Unfortunately, sulphanilamide is not very soluble in water and dissolves very slowly. Rather than add a solution of sulphanilamide to blood and thereby introduce numerous complications, it was added to the blood in finely powdered form. In all probability only part of it dissolved, and the figures for the sulphanilamide concentrations in the following Table are certainly a good deal higher than those actually present.

10 ml. of oxalated venous blood was divided into two equal parts, and one of these was added to finely powdered sulphanilamide which had been deposited inside the tube by evaporation of a solution of the compound in water. The sulphanilamide was persuaded to dissolve as much as possible by gentle mixing. Each specimen was again divided into two parts. The first of each pair was kept in the venous state (which meant working with it as rapidly as possible and avoiding unnecessary shaking): the second of each pair was oxygenated. The fragility of the cells of all four samples was then determined in the usual way in sodium chloride solution.

Six experiments, using different specimens of blood, were carried out.

Table 8. The Effect of Sulphanilamide in inhibiting the action of Carbonic Anhydrase, and preventing the Change in Fragility which occurs on Oxygenation. (S = Sulphanilamide).

Blood Specimen	A	B	C	D	E	F
Concentration of NaCl (g./100 ml.)	0.410	0.395	0.395	0.380	0.380	0.380
Concentration of S. (mg./ml.)	2.5	2.5	1.2	1.2	4.5	2.2
	Percentage Haemolysis					
Venous blood	56	75	55	69	97	86
Venous blood + S	50	69	55	67	96	85
Oxygenated blood	9	21	17	29	52	15
Oxygenated blood + S.	32	55	31	44	79	39

Sulphanilamide has no specific haemolytic or anti-haemolytic effect since the several samples of venous blood show the same degree of haemolysis whether or not sulphanilamide has been added. What slight differences there are, are due to the sulphanilamide increasing the osmotic pressure of the system. Although sulphanilamide does not abolish the oxygenation effect, its presence considerably reduces the magnitude of the change. This is interpreted as indicating that sulphanilamide prevents the loss of carbon dioxide by inhibiting the action of carbonic anhydrase.

The inhibition is by no means complete, but the differences are far beyond the limits of experimental

error. Complete inhibition is hardly to be expected for the decomposition of bicarbonate is not entirely dependent on the presence of the enzyme.

There can be no doubt that the effect of oxygenation is the result of the increase in pH which occurs when carbon dioxide is removed from blood; oxygenation is only one of several ways of bringing this about.

Variation in pH has to be controlled in all biological work - especially in osmotic haemolysis for it causes considerable alteration in the fragility of erythrocytes. Most experimenters in this field have relied on the buffering power of the added plasma to control the pH, and this demands a low ratio of blood to haemolysing system. That a greater degree of buffering capacity is desirable, was first put forward by Simmel (1923) who introduced the haemolysing system referred to on p. 19 which contains, amongst other things, sodium dihydrogen phosphate and sodium bicarbonate. The bulk of the osmotic pressure, however, is due to sodium chloride; of a total of 9.05 g. of several salts dissolved in 1,000 ml. of water to form the stock Simmel solution, sodium chloride contributes 8.2 g. so that the buffering power of this solution is very limited. No other workers have attempted to use buffer solutions and, judging by the literature, Simmel's

solution does not enjoy any great popularity. Nevertheless, it is sound in principle since it attempts to control an important variable. But the difficulties introduced by its complexity more than outweigh its modest buffering power. In particular, there is no simple method of calculating its osmotic pressure.

Others have carried out experiments on osmotic haemolysis using diluted plasma or serum as the haemolysing system - again correct in principle, but still making calculation of the osmotic pressure impossible. And, as will be shown in later sections, the osmotic pressure of the haemolysing system is more important than the concentration.

The observation that oxygenation is merely a convenient method of removing carbon dioxide does nothing to explain why alteration in pH should affect the osmotic resistance. But it is of two-fold importance. Firstly, venous blood is quite unsuitable for these experiments; oxygenation stabilises it and thus excludes one variable. Secondly, it emphasises the importance of pH control.

With sodium chloride solution as the haemolysing system, pH control of the final mixture depends on the buffering power of the added plasma, and this leads to a consideration of the optimal dilution of blood to use in these experiments.

The dilution affects the degree of haemolysis

directly, for the greater the proportion of blood in the mixture, the greater the proportion of added plasma, and the greater the osmotic pressure of the medium in which the erythrocytes are suspended. If the proportion of blood is pushed up sufficiently far, no haemolysis will occur even with the most dilute solution of sodium chloride, and if it be sufficiently far reduced, the buffering power of the system will approximate to zero. A balance must be struck. A dilution of 1 in 20 confers a moderate buffering power on the system without controlling the pH absolutely. And this dilution involves the use of convenient amounts of material such as have already been described in Part 1, and the percentage haemolysis can be determined with an accuracy of about 1%.

Part 3.

The Effect of Changes of pH on Osmotic Resistance.

The experiments in the preceding section make it desirable that the relationship between pH and osmotic fragility be fully examined. At a dilution of 1 in 20, the plasma buffers are not sufficient to control the pH of the system. Since sodium chloride solution has no buffering capacity whatever, experiments designed to have adequate control over the pH must employ buffers and the maximum buffering capacity must be available provided that the osmotic pressure is sufficiently low to allow some degree of haemolysis. The most desirable range of pH is between 7.0 and 8.5 and phosphate buffers are obviously indicated.

Phosphate Buffers.

The phosphate buffer in general use is a mixture of equi-molar solutions of disodium hydrogen phosphate and potassium dihydrogen phosphate. Why the potassium salt should be used in place of sodium dihydrogen phosphate is not at all clear from the literature, except that it has the advantage of crystallising in the anhydrous state. Both the sodium salts contain water of crystallisation and form a series of hydrates having the formulae:-

$Na_2HPO_4.12H_2O$ $Na_2HPO_4.7H_2O$ $Na_2HPO_4.2H_2O$

and $NaH_2PO_4.2H_2O$ $NaH_2PO_4.H_2O$.

The most serious difficulty in the preparation

of this buffer mixture is that the disodium salt is usually found in the form of the dodecahydrate, and this hydrate is markedly efflorescent. Most specimens of it which have been examined contained considerably less than the theoretical percentage of water of crystallisation. On the other hand, all samples of the dihydrate of the monosodium salt which have been obtainable have been found to contain percentages of water of crystallisation within the limits of experimental error.

The following Table shows analyses of the best specimens of the two salts which could be procured.

Table 9. Percentage Water of Crystallisation of the two Sodium Hydrogen Phosphates.

Calculated	60.37%	23.08%
Method of Drying.	$Na_2HPO_4.12H_2O$	$NaH_2PO_4.2H_2O$
P_2O_5 in vacuo; 18°C.	54.70%	23.17%
H_2SO_4 in vacuo; 100°C.	57.84%	23.04%
Oven; 120-130°C.	58.22%	23.16%

These figures are different analyses of 2 g. samples of the same two batches of salts.

There is no danger of losing water of constitution at the temperatures used. According to Mellor (A System of Inorganic Chemistry, Vol. 2) decomposition of the disodium salt to form pyrophosphate does not begin until the temperature is

raised to 230-240°C. Decomposition of the mono-sodium salt to form disodium pyrophosphate ($Na_2H_2P_2O_7$) does not begin until the temperature is raised to 190°C.

The disodium salt cannot be used for the preparation of accurate standard solutions, since different specimens of the same batch contain different percentages of water of crystallisation. (Table 9). This difficulty is not a new discovery. Clark and Lubs (1916) in making up their series of standard buffers, had such an aversion to salts containing water of crystallisation that they declined to use them whether the hydrates were stable or not. In the preparation of phosphate buffers they used potassium dihydrogen phosphate and sodium hydroxide. Naegeli (1926) has met with the same trouble and used a special preparation of the disodium heptahydrate, but he took the precaution of standardising his solutions gravimetrically. Cohn (1927) has discussed the whole problem at length.

According to Clark (1928) the preparation of disodium hydrogen phosphate dihydrate (which contains 20.20% water of crystallisation) consists of "..... exposing to the ordinary atmosphere, the crystals containing 12 moles of water. An exposure of two weeks is generally sufficient". No confirmation of this statement has been found during the present

work. A sample of the dodecahydrate containing 58.0% of water of crystallisation was exposed to the air of the laboratory for 14 days, but at the end of that time it still contained 53.8% of water. And on treatment with dilute hydrochloric acid, a good deal of carbon dioxide, which had been taken up from the atmosphere, was evolved.

There is a further and more important objection to the use of the classical Na_2HPO_4-KH_2PO_4 buffer system. The use of the potassium salt introduces an additional complication, and there is evidence that the potassium ion has a specific effect on the erythrocyte, altering the permeability of the membrane - Davson (1942). Since only low concentrations of potassium occur extracellularly, it would be an obvious advantage if sodium were the only metallic ion present in the haemolysing system.

These results and considerations led to the decision to use a phosphate buffer composed of the two sodium salts. In order to avoid all possible confusion, the anhydrous salts were prepared by heating $Na_2HPO_4.12H_2O$ and $NaH_2PO_4.2H_2O$ at 120-125°C. to constant weight. The anhydrous salts were stored in a desiccator over concentrated sulphuric acid. Both specimens contained small amounts of water which could be removed by heating for prolonged periods at 100°C. over concentrated sulphuric acid; the amounts were:- Na_2HPO_4 (0.01%) and NaH_2PO_4 (0.04%). In the following text, all

weights and concentrations are expressed in terms of the anhydrous salts only.

The Fragility of Erythrocytes in Phosphate Solutions.

As a preliminary, it is necessary that the fragility of normal erythrocytes in solutions of the two sodium phosphates be examined.

Oxalated blood containing the minimum of the anticoagulant was obtained from normal healthy donors. It was fully oxygenated and then

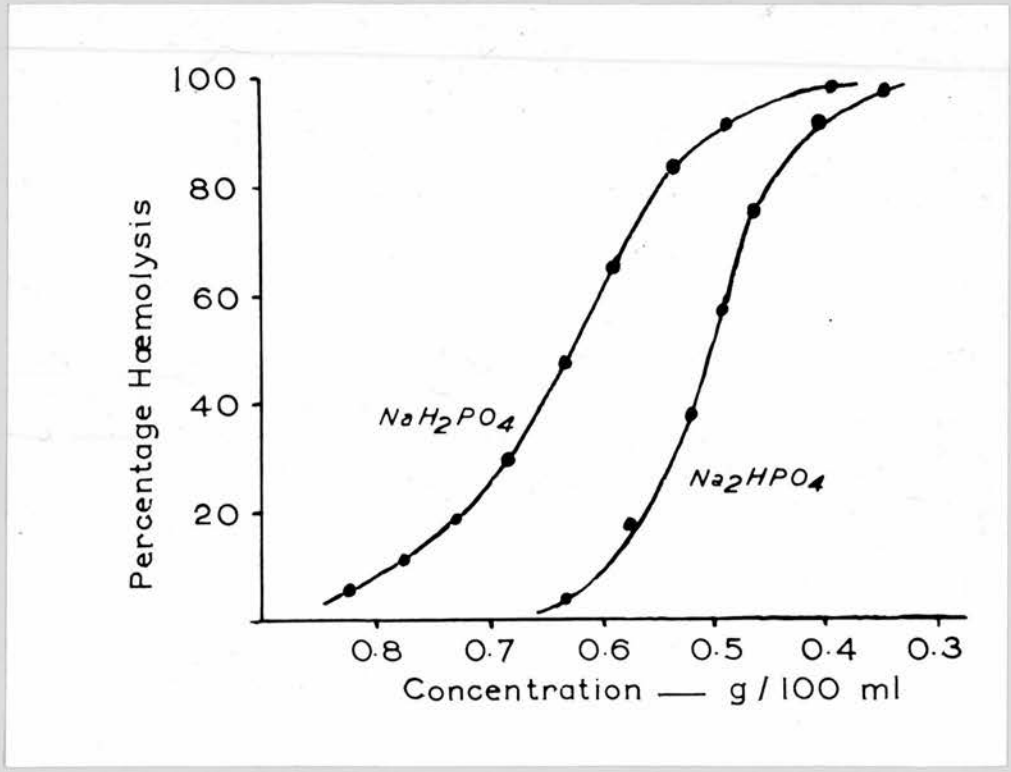

Figure 1. Percentage Haemolysis Curves in Solutions of the two Sodium Hydrogen Phosphates.

distributed into a series of solutions of sodium dihydrogen phosphate (prepared by dilution from a stock reagent), the final dilution of the blood

being 1 in 20. After mixing, the samples were allowed to haemolyse at 20°C. for 1 hour and the percentage haemolysis was determined in the usual way. The fragility curve is obtained by plotting the percentage haemolysis against the concentration of phosphate - see Figure 1. The experiments were repeated using disodium hydrogen phosphate solution as the haemolysing agent. Each of the curves in Figure 1 represents the average of 5 experiments - each using a different specimen of blood. There was little individual variation between the five curves of each series.

Solutions of sodium chloride (vide infra), sodium dihydrogen phosphate and disodium hydrogen phosphate which will produce 50% haemolysis under these conditions of temperature etc., are of different concentrations and have different osmotic pressures. The differences may be due to specific effects of the various ions, but the variation in pH certainly plays an important part. In sodium chloride solution, the pH is about 7.85; in sodium dihydrogen phosphate it is about 5.65; and in disodium hydrogen phosphate it is about 8.5.

The fragility curves shown in Figure 1 are not themselves of much importance, but they indicate the range of concentration at which it is necessary to work.

Iso-osmotic Phosphate Buffer Solutions.

It has already been mentioned that equi-molar solutions of the two sodium hydrogen phosphates are not iso-osmotic, and the preparation of iso-osmotic solutions of these two salts forms the next step in this investigation. There is no reference to this subject in the published literature. If iso-osmotic solutions of these two salts are prepared, then the solutions can be mixed together to give any desired pH between the limits of 4.4 and 8.9 while the osmotic pressure remains constant. As this problem has never apparently been studied before, it will be reported in detail.

Sodium Dihydrogen Phosphate.

This salt ionises primarily as a binary electrolyte according to the equation:

$$NaH_2PO_4 = Na^+ + H_2PO_4^- \quad \text{.....(i)}$$

There is undoubtedly a good deal of further ionisation of the $H_2PO_4^-$ ion according to the equation: $H_2PO_4^- = H^+ + HPO_4^{=}$ .....(ii) for solutions of this salt are acid and have a pH in the region of 4.4.

Lastly, there is the possibility of still further ionisation according to the equation:

$$HPO_4^{=} = H^+ + PO_4^{\equiv} \quad \text{.....(iii)}$$

As will be shown later, the bulk of the ions are derived from equation (i). Hydrogen ions arising

from equation (ii) are present in small concentration; and it is unlikely that the third stage of ionisation proceeds very far.

Disodium Hydrogen Phosphate.

This salt ionises mainly as a ternary electrolyte according to the equation:

$$Na_2HPO_4 = 2Na^+ + HPO_4^= \quad \text{.....(iv)}$$

As before, there is the possibility of further ionisation of the $HPO_4^=$ ion (equation (iii)), but this is most unlikely for the solution is alkaline and has a pH in the region of 8.9.

With this salt, there is the further complication of salt hydrolysis according to the equation:

$$Na_2HPO_4 + 2\,H_2O = 2\,NaOH + H_3PO_4 \quad \text{.....(v)}$$

with ionisation of the sodium hydroxide which is formed.

In a buffer mixture of the two sodium hydrogen phosphates, all the following osmotically active particles are present:-

NaH_2PO_4 ; Na_2HPO_4 ; Na^+ ; $H_2PO_4^-$; $HPO_4^=$; $PO_4^{\equiv}$; H^+ ; OH^-.

Complex as the system appears to be, it is still capable of investigation.

It is unnecessary to embark on a discussion of the modifications which have been introduced into the classical Arrhenius Theory of Ionisation. It will be sufficient at this point to say that the

osmotic pressure of a strong electrolyte may be calculated from the "apparent" degree of ionisation, although modern theory holds that ionisation of a strong electrolyte is always complete, and that experimental deviations from the theoretical values are due to the fact that ionic mobilities depend upon concentration, and that in any solution of finite dilution, interionic forces prevent the complete mobility of the ions. The determination of the "apparent" degree of ionisation, whatever its true interpretation, is essential for calculation of the osmotic pressure, and the expression is therefore used in its original sense.

In determining osmotic pressure of electrolytes, the obvious method of choice is that of depression of the freezing-point which measures the sum-total of the osmotically active particles. Unfortunately, solutions of sodium dihydrogen phosphate and of disodium hydrogen phosphate (especially the latter) undergo considerable super-cooling which introduces an appreciable error by causing an abnormally large amount of ice to crystallise out and so altering the concentration of the liquid phase. It also makes the method tedious in the extreme. Artificial induction of freezing by the addition of a crystal of ice is not to be recommended. The results by this method therefore tend to be a little irregular, and they lack a high degree of reproducibility, nevertheless, the averaging of a sufficient number of

determinations gives figures which are reliable to within about 1%. Conductivity methods are more satisfactory, but require more elaborate apparatus and greater technical precautions. Both methods have been used.

Freezing-Point Depressions.

Depression of the freezing-point measurements, at this stage of the work, were made in the usual way with a Beckmann thermometer accurate to 0.002°C.

(A). Solutions of Sodium Dihydrogen Phosphate.

The following results were obtained:-

Table 10. Depression of the Freezing-Point of aqueous Solutions of Sodium Dihydrogen Phosphate.

Concentration of NaH_2PO_4 (g. per 100 ml.).	Depression of the Freezing-Point.
0.800	0.225°C.
0.700	0.198
0.600	0.170
0.500	0.143
0.400	0.115

(B). Solutions of Disodium Hydrogen Phosphate.

The following results were obtained:-

Table 11. Depression of the Freezing-Point of aqueous Solutions of Disodium Hydrogen Phosphate.

Concentration of Na_2HPO_4 (g. per 100 ml.).	Depression of the Freezing-Point.
0.800	0.252°C.
0.700	0.220
0.600	0.192
0.500	0.162
0.400	0.135

When the depression of the freezing-point is plotted against the concentration, a straight-line graph is obtained for both salts. These quantities are directly proportional to each other over this range of concentration - see Figure 2.

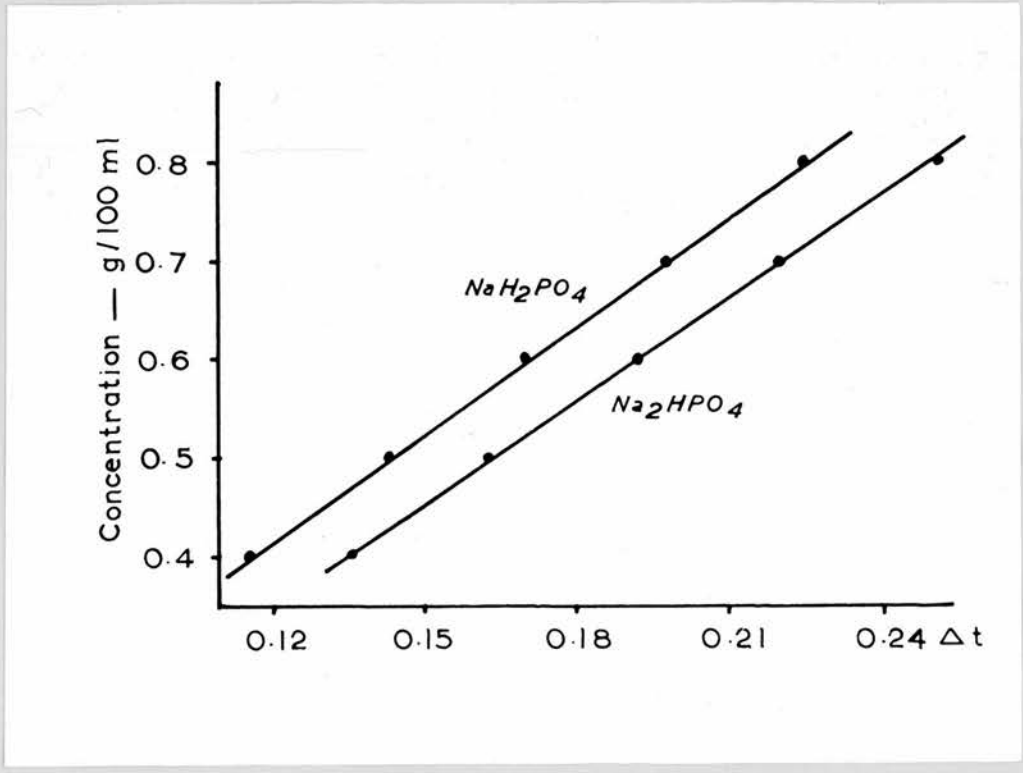

Figure 2. The Relationship between the Depression of the Freezing-Point and the Concentration for the two Sodium Hydrogen Phosphates.

From these graphs can be read off the concentrations of the two salts which will give solutions having the same depression of the freezing-point. When such solutions are mixed together the depression of the freezing-point should remain unaltered provided that the mixture behaves as an ideal mixture. This proved to be the case.

The mixture is not, of course, ideal. The hydrogen ion derived from sodium dihydrogen phosphate (equation (ii)) reacts with the hydroxyl ion derived from the disodium hydrogen phosphate by ionisation of the sodium hydroxide of equation (v) - hence the variation in pH when solutions of these salts are mixed. But the concentrations of hydrogen and hydroxyl ion involved are so small that they cannot be measured by a Beckmann thermometer - a glass electrode is required.

A solution containing 0.732 g. of NaH_2PO_4 is 0.061 M. If it is 85% ionised, then $[Na^+] = [H_2PO_4^-] = 0.052$ N and the total ion concentration from these two sources is 0.104 N. The pH of this solution is about 4.4, hence $[H^+] = 0.000036$ N which is approximately 0.035% of the total ionic concentration. Even allowing for the assumptions in this calculation, the hydrogen ion can contribute very little to the total osmotic pressure. By similar arguments it can be shown that the OH^- derived from Na_2HPO_4 by salt hydrolysis has also an insignificant effect on the total osmotic pressure.

According to accurate graphs drawn from the data given in Tables 10 and 11, the following solutions should have the same freezing-point depression (i.e., are iso-osmotic).

0.514 g. NaH_2PO_4 per 100 ml.)
0.445 g. Na_2HPO_4 per 100 ml.) $\Delta t = 0.147^{\circ}C.$

0.598 g. NaH_2PO_4 per 100 ml.)
0.523 g. Na_2HPO_4 per 100 ml.) $\Delta t = 0.170^{\circ}C.$

0.654 g. NaH_2PO_4 per 100 ml.)
0.576 g. Na_2HPO_4 per 100 ml.) $\Delta t = 0.186^{\circ}C.$

The following Table records the observed depressions of the freezing-point when these pairs of solutions were mixed together.

Table 12. The Depression of the Freezing-Point of Mixtures of Solutions of the Sodium Hydrogen Phosphates.

NaH_2PO_4 Solution. Volume used (ml).	Na_2HPO_4 Solution. Volume used (ml).	Depression of the F.P.	Observed pH.
0.514 g./100 ml.	0.445 g./100 ml.		
100	0	0.151°C	4.50
80	20	0.151	6.16
50	50	0.152	6.73
20	80	0.149	7.34
0	100	0.151	8.97
0.598 g./100 ml.	0.523 g./100 ml.		
100	0	0.173	4.47
75	25	0.170	6.24
50	50	0.171	6.70
25	75	0.170	7.20
0	100	0.171	9.10

0.654 g./100 ml.	0.576 g./100 ml.		
100	0	0.188	4.48
90	10	0.188	5.78
50	50	0.189	6.72
10	90	0.188	7.64
0	100	0.188	9.13

When solutions of these two phosphates, each solution having the same freezing-point, are mixed together in any proportion, the freezing-point of the mixture remains constant. When added together solutions of these salts behave as an ideal mixture within the limits of accuracy of this method.

Conductivity Measurements.

A conductivity bridge operated from a 1,000 cycles/second Cambridge reed hummer was used. The conductivity cell was of the Kohlrausch pattern and was found to have a cell constant of 0.1980 when standardised with potassium chloride solution. All measurements were made in a water thermostat controlled at 25.00 $\pm$ 0.02°C. The water employed for making up all solutions had been re-distilled over permanganate in an all-glass Pyrex distillation apparatus protected from the atmosphere. It had a specific conductivity of 1.68×10^{-6} r.o. For purposes of determining the equivalent conductivity, the equivalent weights of the two salts have been taken as 1/3 NaH_2PO_4 (= 40.03) and 1/3 Na_2HPO_4 (= 47.33).

(A). Solutions of Sodium Dihydrogen Phosphate.

The following results were obtained:-

Table 13. Conductivity Measurements of aqueous Solutions of Sodium Dihydrogen Phosphate.

Concentration of NaH_2PO_4 in g. per 100 ml.	Resistance. (Ohms)	Equivalent Conductivity.
1.100	33.2	21.7
1.000	36.0	22.0
0.900	39.8	22.2
0.800	44.5	22.3
0.700	49.9	22.6
0.600	57.4	23.0
0.500	68.4	23.1
0.400	84.1	23.6

When the equivalent conductivity is plotted against the square root of the concentration, a straight-line graph is obtained (Graph A in Figure 3) and when this graph is produced to zero concentration (i.e. infinite dilution) the equivalent conductivity at infinite dilution is found to be 26.5. This value is assumed, with some justification, to be the correct value provided that the salt continues to ionise as a binary electrolyte. Both in theory and in practice, this value is a purely hypothetical one, for at high dilutions, a different graph is obtained.

Table 14. Conductivity Measurements of aqueous Solutions of Sodium Dihydrogen Phosphate at High Dilutions.

Concentration of NaH_2PO_4. g./100 ml.	Volume in Litres containing 1 gram-eq.	Resistance. (Ohms)	Equivalent Conductivity.
0.0800	50	378	26.1
0.0400	100	735	26.8
0.0160	250	1,760	27.6
0.00800	500	3,470	27.7
0.00400	1,000	6,640	28.2
0.00160	2,500	15,200	28.4
0.000800	5,000	26,400	29.1

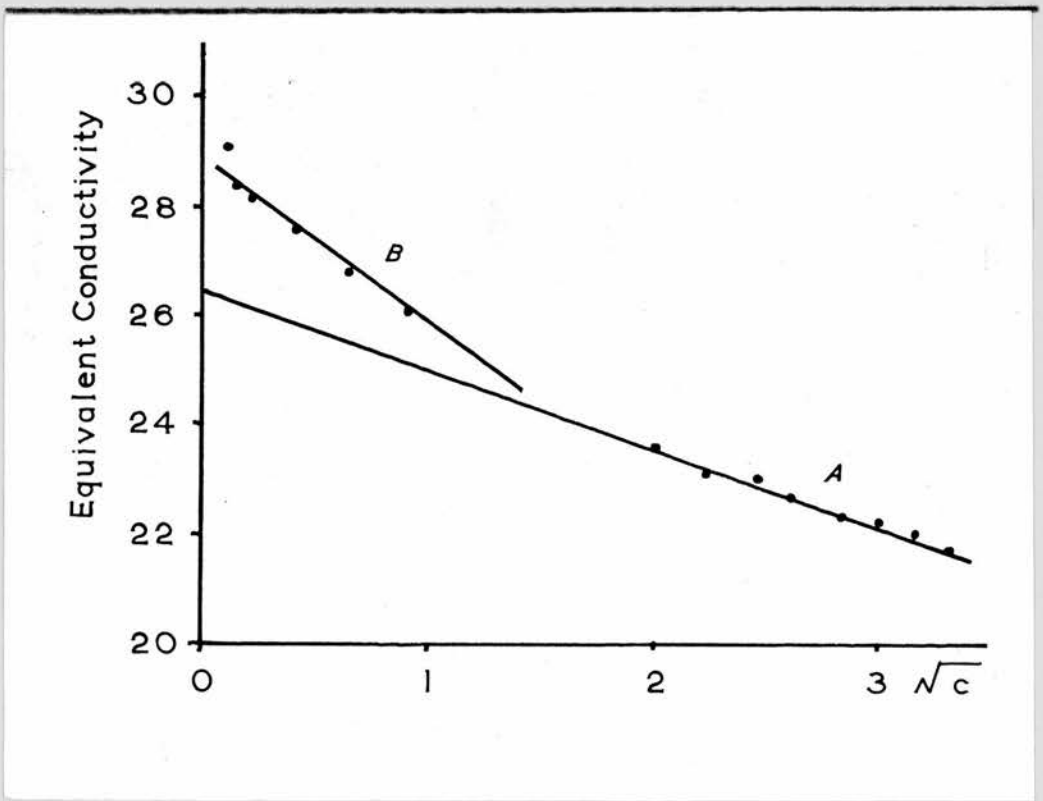

Figure 3. The Equivalent Conductivity of NaH_2PO_4.

When the equivalent conductivity is plotted against the square root of the concentration for dilutions of V = 50 to 5,000 litres, a second linear graph is obtained (Graph B in Figure 3) and the slopes of the two graphs (A and B) are quite different. At these high dilutions, the further ionisation of the $H_2PO_4^-$ ion is evidently becoming significant. At the highest of these dilutions (V = 5,000) there is a suggestion that the last stage of ionisation is beginning.

(B). Solutions of Disodium Hydrogen Phosphate.

The following results were obtained for the equivalent conductivity:-

Table 15. Conductivity Measurements of aqueous Solutions of Disodium Hydrogen Phosphate.

Concentration of Na_2HPO_4 in g. per 100 ml.	Resistance (Ohms)	Equivalent Conductivity.
0.800	24.3	48.3
0.700	27.2	49.2
0.600	30.8	50.6
0.500	35.9	52.3
0.400	43.9	53.3
0.300	56.8	54.9
0.200	82.6	56.5

When the equivalent conductivity is plotted against the square root of the concentration, a

straight-line graph is obtained (Graph A in Figure 4) and when this graph is produced to zero concentration the value of Λ is found to be 65.0. As before, it is assumed that this figure is the theoretical value for the equivalent conductivity at infinite dilution provided that the salt continues to ionise as a ternary electrolyte. As with the monosodium salt, a second graph is obtained at higher dilutions. At these higher dilutions, the following values have been found for the equivalent conductivity.

Table 16. Conductivity Measurements of aqueous Solutions of Disodium Hydrogen Phosphate at high Dilutions.

Concentration of Na_2HPO_4. g./100 ml.	Volume in Litres containing 1 gram-eq.	Resistance. (Ohms)	Equivalent Conductivity.
0.0947	50	161	61.5
0.04733	100	306	64.7
0.00947	500	1,455	67.2

When the equivalent conductivity is plotted against the square root of the concentration for dilutions of 50 - 500 litres, a second linear graph is obtained (Graph B in Figure 4) and the slopes of the two graphs are again different, indicating that at the higher dilutions, the further ionisation of the $HPO_4^=$ ion is becoming significant.

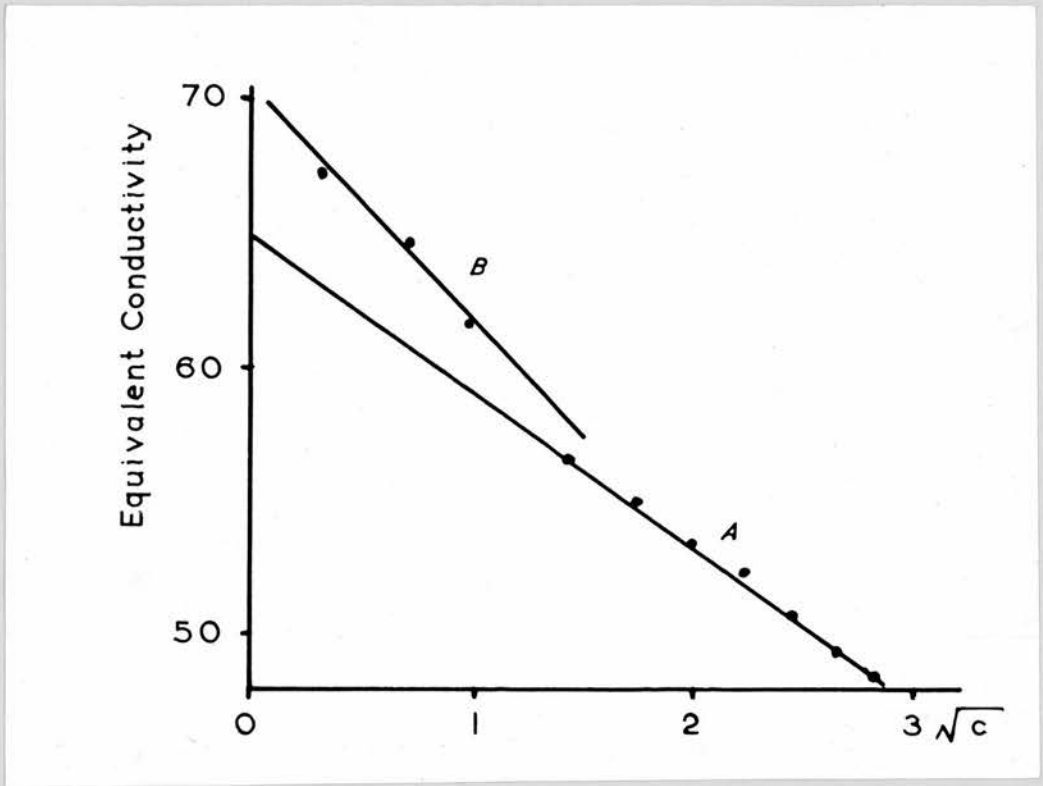

Figure 4. The Equivalent Conductivity of Na_2HPO_4.

At still higher dilutions than those used in Table 16, the equivalent conductivity begins to fall off rapidly due to the absorption of carbon dioxide from the atmosphere during manipulation, causing a relatively great alteration in the conductivity. Since the object of this experiment has been achieved - the obtaining of the figure 65.0 for the equivalent conductivity of disodium hydrogen phosphate at infinite dilution - measurements at these very high dilutions were not pursued.

When the concentrations of these salts are plotted against the measured resistances (from the data given in Tables 13 and 15) two curves are obtained (see Figure 5, p. 48).

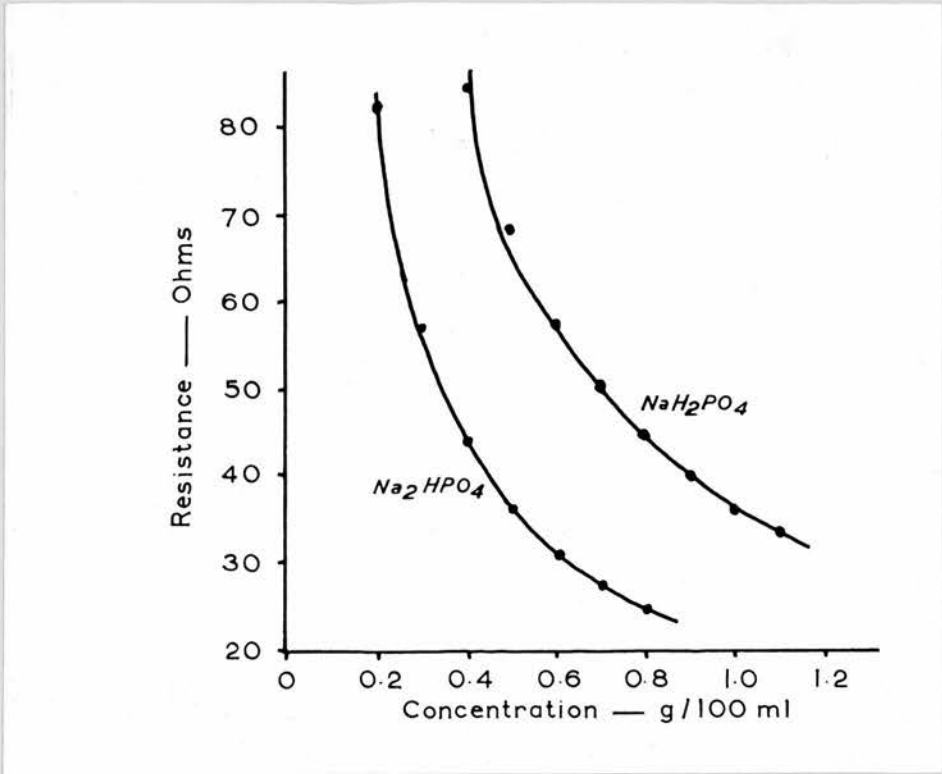

Figure 5. The Relationship between Electrical Resistance and Concentration for the two Sodium Hydrogen Phosphates.

From these graphs can be read off the concentration of each salt required to produce a solution of any selected resistance. For example, the following pairs of solutions should have the same resistance:-

0.732 g. NaH_2PO_4 per 100 ml.)
0.361 g. Na_2HPO_4 per 100 ml.) } ω = 48.0 ohms.

0.944 g. NaH_2PO_4 per 100 ml.)
0.469 g. Na_2HPO_4 per 100 ml.) } ω = 38.0 ohms.

The following Table (p. 49) records the observed resistances when these pairs of solutions are mixed together in varying proportions.

When solutions of these two phosphates, each having the same conductivity, are mixed together in

any proportion, the conductivity remains constant, i.e., when added, solutions of these two salts behave as an ideal mixture.

Table 17. The Electrical Conductivity of Mixtures of Solutions of the two Sodium Hydrogen Phosphates.

NaH_2PO_4 Solution. Volume used (ml.)	Na_2HPO_4 Solution. Volume used (ml.)	Observed Resistance. (ohms)	Observed pH.
0.732 g./100 ml.	0.361 g./100 ml.		
250	0	48.0	4.44
200	50	48.5	5.87
150	100	48.5	6.30
100	150	48.3	6.66
50	200	48.5	7.09
0	250	48.6	8.83
0.944 g./100 ml.	0.469 g./100 ml.		
250	0	38.4	4.41
200	50	38.3	5.86
150	100	38.8	6.30
100	150	38.9	6.65
50	200	38.6	7.09
0	250	38.7	8.85

If these two salts behave ideally when their solutions are mixed, it could be argued that, if we add 100 ml. of Na_2HPO_4 solution to 100 ml. of NaH_2PO_4 solution (both having the same conductivity) then the NaH_2PO_4 solution has been diluted with 100

ml. of water (containing, incidentally, some Na_2HPO_4) and hence the equivalent conductivity should be disproportionately increased since the degree of ionisation increases with increasing dilution, and vice versa. But it must be remembered that both salts have the sodium ion in common and its presence in the added Na_2HPO_4 solution will prevent any appreciable increase in the degree of ionisation of the NaH_2PO_4 - the "common ion effect". The sodium concentration is of the same order in solutions of the two salts which have the same conductivity.

Calculation of the Osmotic Pressure.

According to classical theory, the calculation of the osmotic pressure of a solution of an electrolyte requires a knowledge of the degree of ionisation and this has been obtained from the conductivity measurements by the equation:

$$\alpha = \Lambda / \Lambda_\infty \quad \text{.....(vi)}$$

and from freezing-point depression measurements by the equation:

$$\Delta t = K \cdot \frac{w}{M} \cdot (1 + \alpha) \quad \text{.....(vii)}$$

for a binary electrolyte such as NaH_2PO_4, or by:

$$\Delta t = K \cdot \frac{w}{M} \cdot (1 + 2\alpha) \quad \text{.....(viii)}$$

for a ternary electrolyte such as Na_2HPO_4.
The following Table (Table 18, p. 51) gives the values calculated for the degree of ionisation of these two salts from the data set out in Tables 10,

11, 13 and 15. From these values, the osmotic pressure may be calculated from the equation:

$$\Pi \cdot V = (1 + \alpha) \cdot R.T \quad \text{.....(ix)}$$

for a binary electrolyte, or the equation:

$$\Pi \cdot V = (1 + 2\alpha) \cdot R.T \quad \text{.....(x)}$$

for a ternary electrolyte.

Table 18 A. The Degree of Ionisation and the Osmotic Pressure (in atmospheres) for Solutions of Sodium Dihydrogen Phosphate.

Conc. of NaH_2PO_4 g. per 100 ml.	α from Conductivity.	α from F.P. Depression	Π from Conductivity.	Π from F.P. Depression.	Average value of Π
1.100	0.82	-	4.08	-	-
1.000	0.83	-	3.73	-	-
0.900	0.835	-	3.36	-	-
0.800	0.84	0.82	3.00	2.97	2.99
0.700	0.85	0.825	2.64	2.61	2.63
0.600	0.865	0.83	2.28	2.24	2.26
0.500	0.87	0.845	1.91	1.88	1.89
0.400	0.89	0.855	1.54	1.52	1.53

Table 18 B. The Degree of Ionisation and the Osmotic Pressure (in atmospheres) for Solutions of Disodium Hydrogen Phosphate.

Conc. of Na_2HPO_4 g. per 100 ml.	α from Conductivity.	α from the F.P. Depression	π from Conductivity	π from the F.P. Depression	Average value of π
0.800	0.74	0.705	3.42	3.32	3.37
0.700	0.755	0.705	3.02	2.91	2.97
0.600	0.78	0.72	2.65	2.52	2.59
0.500	0.805	0.74	2.25	2.14	2.20
0.400	0.82	0.79	1.82	1.78	1.80
0.300	0.845	—	1.39	—	—
0.200	0.87	—	0.945	—	—

There is a linear relation between concentration and osmotic pressure over the range described for each salt in Tables 18 A and B. From the graphs obtained when these quantities are plotted against each other (Figure 6, p. 53) there can be read off the concentrations of each salt required to give iso-osmotic solutions.

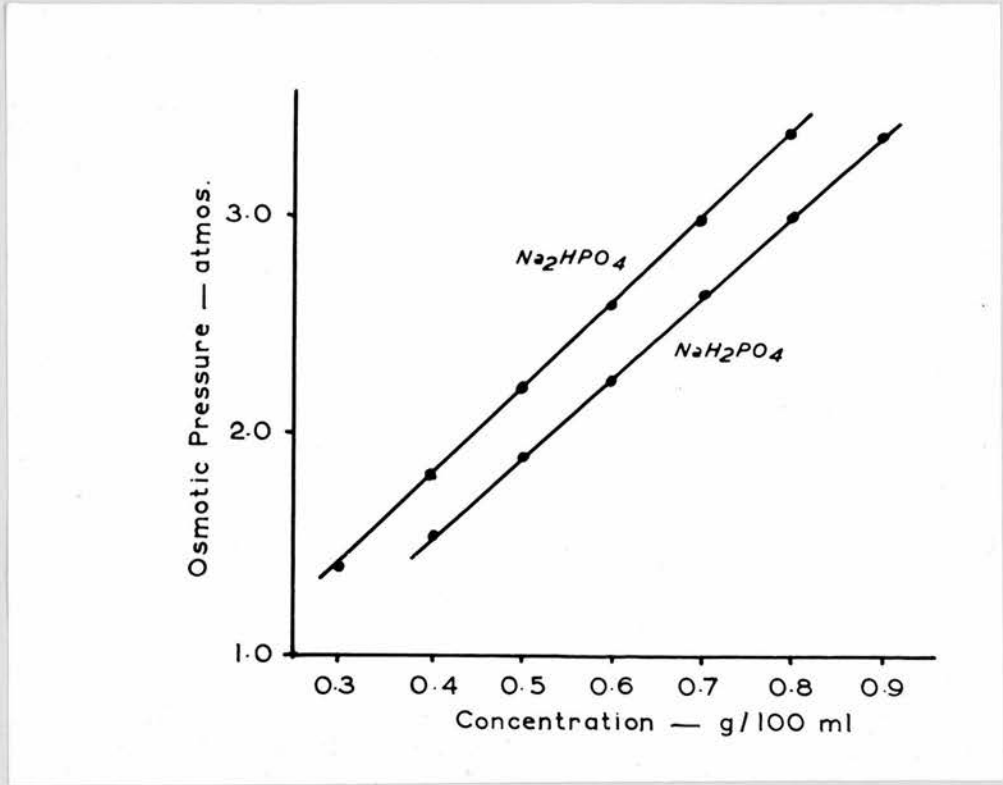

Figure 6. The Relation between Concentration and Osmotic Pressure for Solutions of the two Sodium Hydrogen Phosphates.

The figures in Table 19 (p. 54) have been obtained from large-scale graphs using the average values given in Tables 18 A and 18 B. Buffer solutions may be made up by taking different volumes of any of these pairs of standard solutions - the pH range covered lying between 4.4 and 8.9. Such buffers will be iso-osmotic whatever the proportions of the two solutions.

Table 19. Concentrations of the Sodium Hydrogen Phosphates required to prepare Iso-Osmotic Solutions at 20°C. Concentrations in g. per 100 ml.

Osmotic Pressure. (atmospheres).	Concentration of NaH_2PO_4.	Concentration of Na_2HPO_4.
3.00	0.802	0.706
2.80	0.748	0.655
2.60	0.694	0.603
2.40	0.639	0.551
2.20	0.584	0.500
2.00	0.530	0.449
1.80	0.475	0.398

As a side-issue, one can plot (Figure 7, p. 55) the volume of any one of the two buffer solutions (say sodium dihydrogen phosphate) against the pH for all the protocols in Table 12 (p. 41) irrespective of the osmotic pressure. All points lie on a sigmoid curve since the pH depends only on the ratio of the two salts. This curve is useful in the selection of any desired pH.

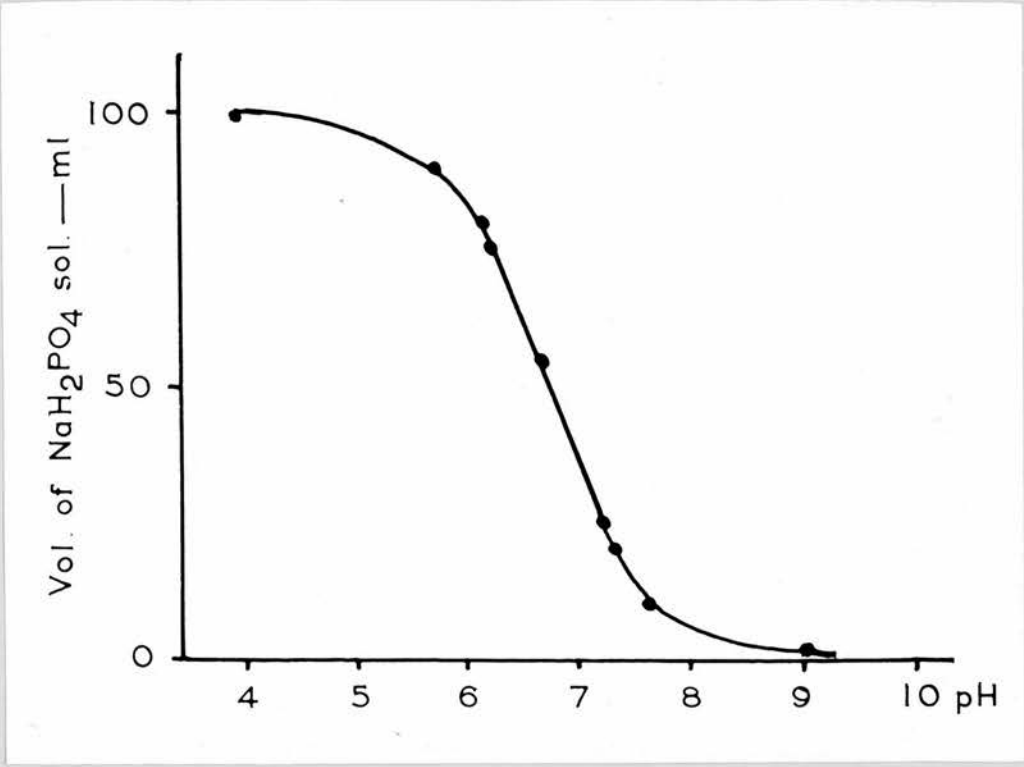

Figure 7. The Relationship between the Volumes of Sodium Dihydrogen Phosphate used and the pH.

Haemolysis at Constant Osmotic Pressure.

When blood is added to one of these phosphate buffer mixtures to a final dilution of 1 in 20, the pH of the mixture changes due to the different pH of the blood and due to the buffering power of the blood. The actual pH of the blood-buffer mixture must be determined experimentally in each case. The buffering power of a solution of sufficiently low osmotic pressure to produce a measurable degree of haemolysis is not sufficiently great to prevent a change of pH when blood is added to it in this dilution; and the further the pH of the buffer from the pH of the blood, the greater will be the alteration.

In the first experiments, solutions of the two

phosphates, each having an osmotic pressure of 2.40 atmospheres, were prepared and mixed in varying proportions. 0.500 ml. of oxygenated blood was added to 9.50 ml. of buffer and the mixture allowed to stand for 1 hour at 20°C. The pH and the degree of haemolysis were then determined. Seven such experiments were carried out using the same set of buffers, and the results were averaged:-

Table 20. Percentage Haemolysis in Iso-Osmotic Phosphate Buffer. 7 Samples of normal, oxygenated blood were used. Π = 2.40 atmos. T = 20°C.

Vol. NaH_2PO_4 (ml.)	90	70	60	50	40	30	10
Vol. Na_2HPO_4 (ml.)	10	30	40	50	60	70	90
pH of the blood-buffer mixture.	5.99	6.39	6.55	6.71	6.86	7.02	7.53
Percentage haemolysis.	40.7	44.1	47.4	50.1	49.4	39.0	14.8

When the percentage haemolysis is plotted aginst the pH of the blood-buffer mixture, a graph is obtained (see Figure 8, p. 57) which shows (i) a maximum haemolysis in the region of pH = 6.8, and (ii) lesser degrees of haemolysis on either side of this maximum with the curve falling away much more steeply on the alkaline side of neutrality.

As the osmotic pressure is increased to about 2.80 atmospheres, the degree of haemolysis at all pH values approximates to zero and the effect of alteration of the pH is correspondingly reduced.

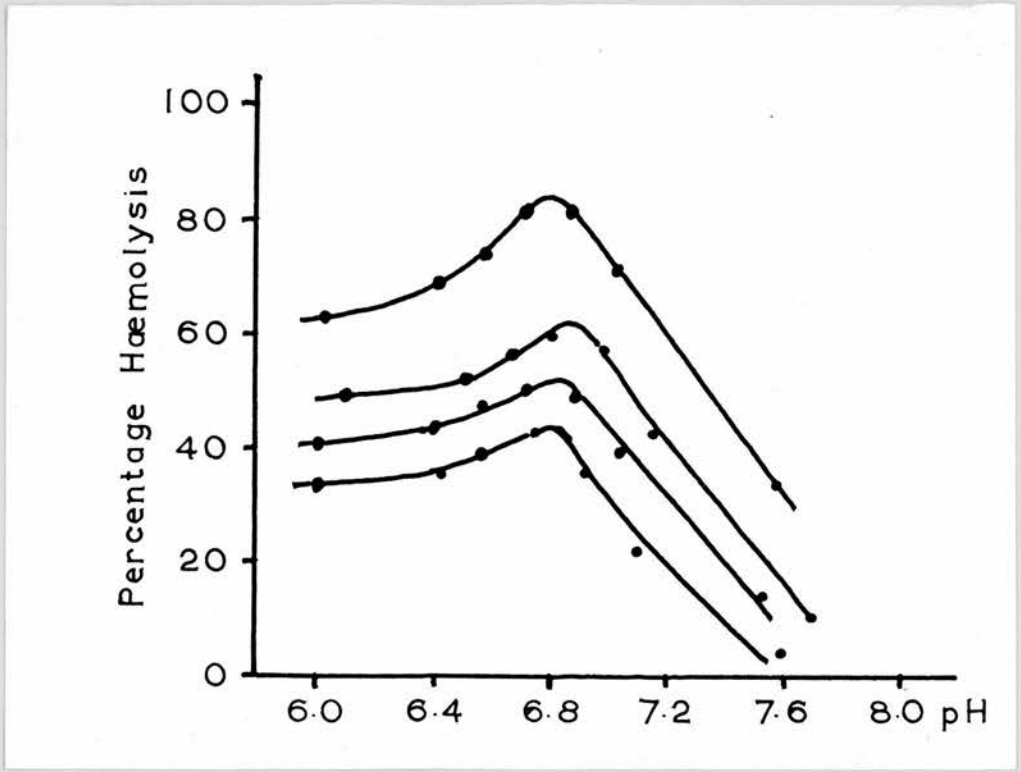

Figure 8. The Effect of pH on the Degree of Haemolysis at Constant Osmotic Pressure. Top curve, Π = 2.30, second, Π = 2.35, third, Π = 2.40 and bottom curve, Π = 2.50 atmos.

If the osmotic pressure is reduced to about 2.20 atmospheres, the degree of haemolysis approximates to 100% at all pH values, and again the pH effect vanishes. Between these two limits, graphs such as those shown in Figure 8 are found. (The graphs corresponding to osmotic pressures of 2.30, 2.35, and 2.50 atmospheres have been drawn from the mean values obtained by the use of five different, normal, blood samples at each osmotic pressure).

The effect of alteration of pH on the volume of the erythrocyte has been investigated by Hampson and Maizels (1926). They have shown that the red cell has a maximal volume at pH 5.4 and their graphs

have the same general form as those in Figure 8. Their paper gives little information on the nature of the medium in which the cells were suspended and it is impossible, from the data given, to judge whether iso-osmotic solutions were used. However, since the volume of the cell and the process of haemolysis are known to be closely related, it is fair to assume that the two methods of approach lead to the same general conclusion viz, that haemolysis (or cell volume) is closely dependent on pH and that the curve shows an unmistakable maximum.

In all twenty-two curves obtained by plotting pH against the degree of haemolysis, the maximum degree of haemolysis was found to lie between pH 6.75 and 6.85. It is tempting to conclude that this maximum is related to the iso-electric point of haemoglobin which is variously quoted in the literature as lying between 6.65 for oxyhaemoglobin and 6.85 for reduced haemoglobin, but this remains to be proved. The suggestion leads to the possibility that the iso-electric state of the protein may be an important factor in haemolysis.

It has already been shown (p. 21) that the effect of oxygenation (a decrease in fragility) can be eliminated by the use of iso-molar buffers which control the pH. A more refined experiment using iso-osmotic buffer solutions and eliminating the potassium, is recorded in Table 21 (p. 59). These

results were all obtained with one single specimen of blood - the experiments being carried out simultaneously. They lead to the same conclusion as the preliminary experiment on this point (cp. Table 7, p. 21).

Table 21. The Haemolysis of a single Specimen of blood (Venous and Oxygenated) in Iso-Osmotic Phosphate Buffer Solutions.

Π = 2.40 atmos. T = 21°C.

Oxygenated Blood.		Venous Blood.	
pH of the Blood-Buffer Mixture.	Percentage Haemolysis.	pH of the Blood-Buffer Mixture.	Percentage Haemolysis.
5.99	57%	5.92	57%
6.40	61%	6.40	59%
6.58	65%	6.53	65%
6.72	72%	6.68	70%
6.87	78%	6.87	78%
7.06	70%	7.05	72%
7.54	40%	7.51	37%

Note: The various haemolysing solutions used in compiling the above Table were made up by mixing various proportions of iso-osmotic solutions (Π = 2.40 atmos.) of the two phosphates - hence the variation in pH.

These figures argue against the iso-electric point being a factor concerned directly in haemolysis. Oxyhaemoglobin and reduced haemoglobin have different base-binding capacities (see Hastings,

Van Slyke, Neill, Heidelberger and Harington (1924), and Van Slyke, Hastings, Heidelberger and Neill (1922)), and therefore have different iso-electric points. These values according to Peters and Van Slyke (1931) are: 6.80 for reduced haemoglobin and 6.65 for oxyhaemoglobin. In the above experiment, the pH is controlled within very narrow limits so that, at any one pH value, reduced haemoglobin and oxyhaemoglobin have the chance to behave differently in fragility experiments, but they do not appear to accept the opportunity, for the degree of haemolysis is practically identical for venous and oxygenated blood. Nevertheless, there is the undoubted conclusion that the maximum degree of haemolysis coincides with the iso-electric point of the protein.

The Relationship between the Fragility of Red cells, the Temperature, and the pH.

Control of the pH does not abolish the increase in the degree of haemolysis which occurs when the temperature is lowered. The figures in the following Table show the effect of change of temperature.

Table 22. Haemolysis of Oxygenated Blood in Phosphate Buffers at Different Temperatures.

Π = 2.50 atmos. at 21°C.

pH of Blood-Buffer mixture.	6.57	6.73	6.83	7.06
Percent haemolysis at 21°C.	40	42	40	29
Percent haemolysis at 10°C.	65	68	62	55

Jacobs and Parpart (1931) concluded that the change in the degree of haemolysis attending change of temperature is associated with the alteration in the relative base-binding power of haemoglobin which alters with temperature - the lower the temperature, the lower the base-binding capacity, and vice versa (see Stadie and Martin (1924)).

When erythrocytes are suspended in one of these phosphate buffers, the pH remains constant; the observed variation, if any, is never greater than 0.02 unit. Hence the base-binding power of haemoglobin in that particular buffer must remain equally constant. Yet the figures in Table 22 show that at these pH values there is a considerable variation in the degree of haemolysis when the temperature is lowered.

These figures are equally at variance with the theory of Jarisch (1921) who put forward the explanation that osmotic haemolysis decreases with rise in temperature due to a change in the hydrogen ion concentration. The pH of a phosphate buffer varies very little with the temperature: a change of temperature from 5° to 37°C alters the pH of these buffers by about 0.05 unit. This change will produce a very small alteration in the degree of haemolysis.

The whole question of the change in the degree of haemolysis brought about by change in temperature is fully discussed in Part 5.

Part 4.

Haemolysis of Erythrocytes in Solutions of the Alkali Halides.

Having dealt with the effect of oxygenation and of change in pH, it is possible to proceed to a brief study of the fragility of erythrocytes in solutions of the alkali halides.

There are many factors other than oxygenation and pH to be considered, and the great diversity of results obtained by different experimenters is due to the choice of diverse conditions under which the fragility has been measured. No two appear to have selected the same combination. The red cell fragility test as described in textbooks of haematology is sufficient to give an indication of normal or abnormal fragility, but it cannot be described as a quantitative estimation in spite of the fact that standard solutions of sodium chloride are used. It is nothing more than a qualitative test since the degree of haemolysis is not determined, and it is quite impossible with any degree of accuracy to decide where haemolysis "begins" or where it "ends". In order to make it quantitative, it is necessary to estimate the degree of haemolysis in a series of salt concentrations and study the curve obtained by plotting the degree of haemolysis against the concentration (or, better, osmotic pressure).

The following factors have to be taken into

account:-

(1) Anticoagulant. There is no reliable evidence that either traces of oxalate or heparin have any specific haemolytic or antihaemolytic properties. Both have been used extensively in the present work over a period of years without any indication that they are other than interchangeable - always provided that the minimal quantity of oxalate is used. Fluoride is said to have a specific antihaemolytic effect.

(2) Oxygenation. This factor has already been discussed in Part 2.

(3) The Nature of the Haemolysing System. Sodium chloride solutions are the most obvious choice since this salt is the main contributor to the total osmotic pressure of plasma. As far as can be ascertained, neither the sodium nor the chloride ion has any specific lytic action; both occur in the plasma in high concentration; and neither affects the pH. The main drawback to sodium chloride solution is its lack of buffering power.

The great majority of studies on fragility have employed solutions of sodium chloride. Rarely, other electrolytes and such non-electrolytes as sucrose have been used. Some prefer to use diluted plasma and Simmel (1923) introduced a very complicated haemolysing system which has been referred to above (pp. 19, 26).

(4) Dilution of the Blood. This factor has already been mentioned (see pp.27-28). The effect of varying the dilution (other factors being kept constant) is shown by the following data:-

Dilution, 1 in 10: Degree of Haemolysis = 27%

Dilution, 1 in 20: Degree of Haemolysis = 60%

Dilution, 1 in 40: Degree of Haemolysis = 89%.

One must therefore strike a balance between the extremes; a dilution of 1 in 20 has a moderate buffering power due to the plasma, and it involves quantities which can readily be measured.

This variation in the dilution of the blood is one item of many which accounts for the disparity of the results of different workers. Consider, for example, the following medley of dilutions which appear in the literature:-

Kato (1941)	Dilution = 1 in 3.
Olbrich (1947)	1 in 20.
Dacie and Vaughan (1938)	1 in 23.
Creed (1938)	1 in 26.
Bohr (1946)	1 in 26.
Daland and Worthley (1934-35)	1 in 37.
Whitby and Hynes (1935)	1 in 200.
Hunter (1940)	1 in 201.

(5) Temperature. This factor is fully discussed in Part 5. All observers are agreed that the lower the temperature, the greater the degree of haemolysis, and vice versa.

Ideally, haemolysis should be allowed to proceed at 37°C. in a constant temperature room with specially warmed equipment, etc. The technical difficulties would be considerable. At the other extreme, there seems to be no advantage in working in the region of 0°. The use of "room temperature" seems the obvious choice, provided that the actual temperature is stated.

The considerable variation in percentage haemolysis on changing the temperature is shown by the following figures:-

	Percent Haemolysis.		
	8°	19°	37°
Blood A.	82%	75%	43%
Blood B.	75%	56%	17%

Few authors state the temperature around which they work:-

Kato (1941)	Not stated.
Olbrich (1947)	Not stated.
Dacie & Vaughan (1938)	3°C.
Creed (1938)	Not stated.
Bohr (1946)	"Refrigerator temperature".
Daland & Worthley (1934-35)	"Refrigerator temperature".
Whitby & Hynes (1935)	Not stated.
Hunter (1940)	"Room temperature".

(6) Other factors. It is well known that collection of blood by needle and syringe results in a loss of carbon dioxide and a shift in pH. Oxygenation

removes the rest of the carbon dioxide, so that losses during venepuncture need not be considered.

The accuracy of the method of determining the degree of haemolysis should be above suspicion. Many workers, for instance, use the "drop" as a (presumably) accurate measure of volume. Such work deserves little consideration.

Human blood has been used throughout the present work. The chief variation encountered is the slight variation from one person to another, and in the same person from day to day, of the ratio of cells to plasma, and this directly affects the degree of haemolysis by affecting the amount of plasma in the haemolysing medium. The variation is small, and its effect is small, but this may be sufficient to account for the differences in the degrees of haemolysis of different blood specimens.

In order to standardise the determination of the cell fragility, it was decided to adhere to the following conditions unless otherwise stated:-

(a) Human blood from normal healthy subjects,
(b) Anticoagulant: Heller & Paul's oxalate, or heparin, in minimal amount,
(c) Blood to be fully oxygenated,
(d) Dilution to be 1 in 20,
(e) The reference haemolysing system to be sodium chloride solution,
(f) Temperature: 20°C.,
(g) Duration of haemolysis: 1 - 2 hours,
(h) Method: Determination of liberated haemoglobin.

Parpart and his colleagues (1947) have emphasised the lack of accuracy caused by visual selection of the salt concentrations at which haemolysis "begins" and "ends", pointing out that, in these extreme regions of the haemolysing curve, small differences in the degree of haemolysis correspond to large differences in the salt concentration. It is therefore proposed to consider only the region between 10% and 90% haemolysis, for even with an accurate method of determining the degree of haemolysis it is still impossible to decide the exact points where lysis begins or ends.

Haemolysis in Sodium Chloride Solution.

Figure 9 (p. 68) shows the results of two series of haemolysis curves; one of venous and the other of oxygenated blood. 20 blood samples were used for the determination of each curve.

Both curves are sigmoid, the curve for venous blood showing a "shift to the left" - the cells being more fragile than those of oxygenated blood due to the lower pH.

In addition to determining the average values of sodium chloride concentration corresponding to 10%, 50% and 90% haemolysis, it is also necessary to determine the slope of the curve so that one may distinguish between a blood which haemolyses over the normal range and one which haemolyses over an abnormally large range. The simplest method is to

consider the difference in the sodium chloride concentrations corresponding to 10% and 90% haemolysis; this is referred to as the "span" of the curve.

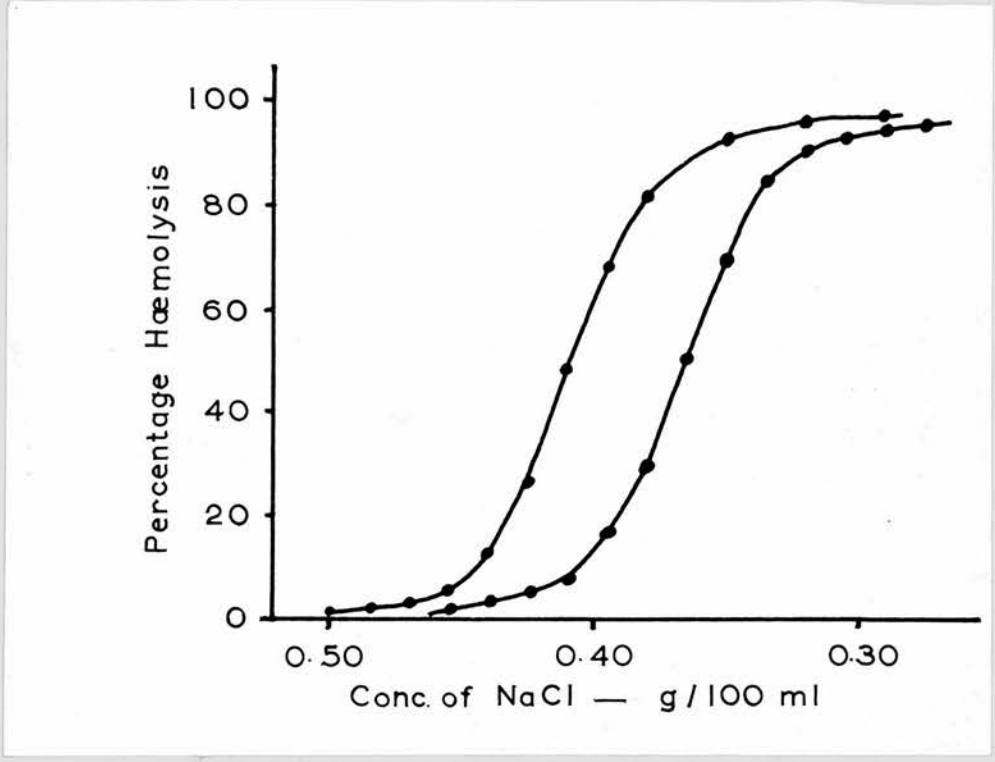

Figure 9. Haemolysis Curves of Venous and Oxygenated Blood in Solutions of Sodium Chloride. On the left: venous blood. On the right: oxygenated blood.

The individual variation is shown in the following Table:-

Table 23. The Haemolysis of Venous and Oxygenated Blood in Sodium Chloride Solution at 20°C.

	Venous Blood		Oxygenated Blood.	
	Conc. NaCl g./100 ml.	Standard Deviation	Conc. NaCl g./100 ml.	Standard Deviation
10% Haemolysis	0.440	0.0144	0.395	0.0137
50% Haemolysis	0.409	0.0177	0.366	0.0135
90% Haemolysis	0.373	0.0190	0.332	0.0141
"Span"	0.067	0.0127	0.063	0.0066

Sigmoid Haemolysis Curves.

All osmotic haemolysis curves i.e., curves obtained by plotting degree of haemolysis against tonicity, are of the same sigmoid type as those shown in Figure 9. No satisfactory explanation has yet been put forward to explain the shape of this curve. It has been suggested that the fragility of the individual cells depends on their age, the older cells haemolysing in the more concentrated solutions, the younger cells being more resistant (Daland and Worthley (1935), Mermod and Dock (1935), Stephens (1940)). On the other hand, Whitby and Hynes (1935) find that reticulocytes have the same fragility as mature erythrocytes. It has also been suggested by Castle and Daland (1937) that differences in the susceptibility of cells to hypotonic saline is due to differences in cell form and not to differences in osmotic behaviour;

and by Davson (1943) that erythrocytes may contain different concentrations of osmotically active material and so haemolyse at different (external) osmotic pressures. From a study of these publications, the most attractive explanation is that the fragility of the membrane is a function of the age of the cell - the older the cell, the less able is it to stand up to changes in osmotic pressure. This diminished resistance need not be a change of any great magnitude, for the normal erythrocyte can exert very little resistance to an osmotic force except to allow water to enter the cell in an attempt to reduce the internal osmotic pressure to the level of the external pressure.

Haemolysis in other Alkali Halides.

The purest available specimens of the various salts were used. If the purity were in any doubt the salt was recrystallised from water. Standard solutions cannot always be made up directly; lithium chloride is said to be the most deliquescent salt known to chemistry and sodium iodide suffers from the same defect. Concentrated solutions of these salts were made up and standardised gravimetrically with silver nitrate; working solutions were prepared by dilution and their concentrations re-checked. With the other salts, the anhydrous compounds were prepared and used.

In calculating osmotic pressures, the necessary data was taken from the International Critical

Tables (1929). The values for both freezing-point depression and conductivity have been examined. There is good agreement of the osmotic pressure when calculated from these protocols - sodium fluoride being the only exception; freezing-point depression values being 7% higher than conductivity values. The data for sodium iodide over the required range are somewhat scanty, but for all the other salts, the data are sufficient and the two methods are in good agreement.

The conditions under which fragility curves have been determined are summarised above (see p. 66). In the following Table there was very good agreement amongst a series of haemolysis curves in solutions of a given salt.

Table 24. Haemolysis Curves of normal, oxygenated, Human Blood (oxalated) at 19 - 20°C. Dilution = 1 in 20. Time of Haemolysis = 1 hour. Results expressed in terms of g. per 100 ml. of the anhydrous salts.

Salt.	Number of Curves.	Percentage Haemolysis. 10%	30%	50%	70%	90%
LiCl	5.	0.276	0.262	0.253	0.245	0.231
NaF	5.	0.332	0.313	0.299	0.286	0.246
NaCl	20.	0.396	0.376	0.366	0.354	0.332
NaBr	5.	0.698	0.652	0.633	0.614	0.577
NaI	5.	0.984	0.923	0.891	0.859	0.796
KCl	8.	0.540	0.518	0.500	0.485	0.449
KBr	5.	0.825	0.787	0.766	0.745	0.699
KI	5.	1.121	1.066	1.031	0.997	0.917
RbCl	5.	0.861	0.818	0.799	0.775	0.721
CsCl	5.	1.159	1.085	1.046	1.011	0.944

In the following Table (Table 25, p. 73) these concentrations have been converted into terms of osmotic pressures. Over the small range of concentration concerned, the osmotic pressure is proportional to the concentration for all practical purposes, since the change in the degree of ionisation over this range is very small. The osmotic pressures have been calculated by equation (ix) p. 51, the value for α being taken as the average calculated from freezing-point and conductivity

measurements from the data in the International Critical Tables.

Table 25. Haemolysis Curves as described in Table 24. Results expressed in terms of atmospheres of osmotic pressure.

	Percentage Haemolysis.				
Salt.	10%	30%	50%	70%	90%
LiCl	2.92	2.77	2.67	2.59	2.44
NaF	3.56	3.35	3.20	3.06	2.63
NaCl	3.04	2.89	2.81	2.72	2.55
NaBr	3.06	2.86	2.78	2.69	2.53
NaI	3.00	2.81	2.72	2.62	2.43
KCl	3.25	3.12	3.01	2.92	2.70
KBr	3.11	2.97	2.88	2.81	2.64
KI	3.04	2.89	2.80	2.70	2.49
RbCl	3.14	2.98	2.92	2.83	2.63
CsCl	3.07	2.88	2.77	2.68	2.50
Average.	3.12	2.95	2.86	2.76	2.55

This series is too small to analyse statistically, but it is obvious that the difference from the average is very small except in the case of sodium fluoride and to a lesser extent, potassium chloride. In the column corresponding to 50% haemolysis, the average difference from the mean is 0.12 atmos. and this represents a variation of only 4.2%. If the result for sodium fluoride be excluded, this

variation is reduced to 2.8% and is probably not significant - using the word significant in its ordinary meaning.

What differences there are between the ions is better shown in the following Figures (pp. 74, 75).

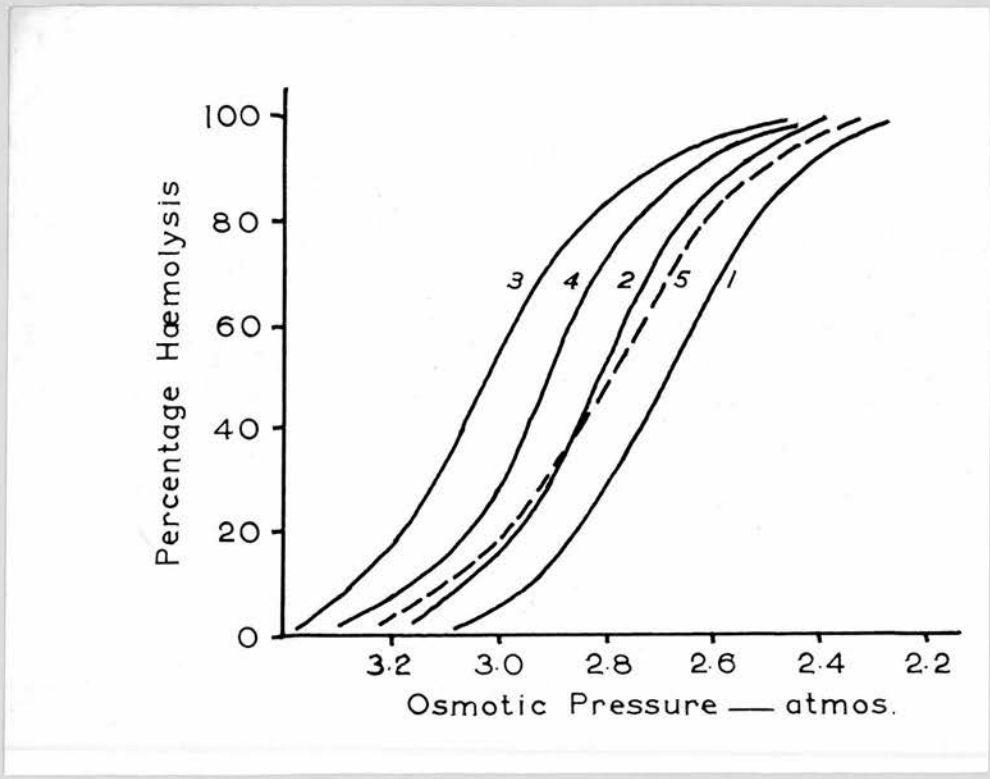

Figure 10. Haemolysis Curves of the Alkali Chlorides.

1 - LiCl 2 - NaCl 3 - KCl 4 - RbCl

5 - CsCl

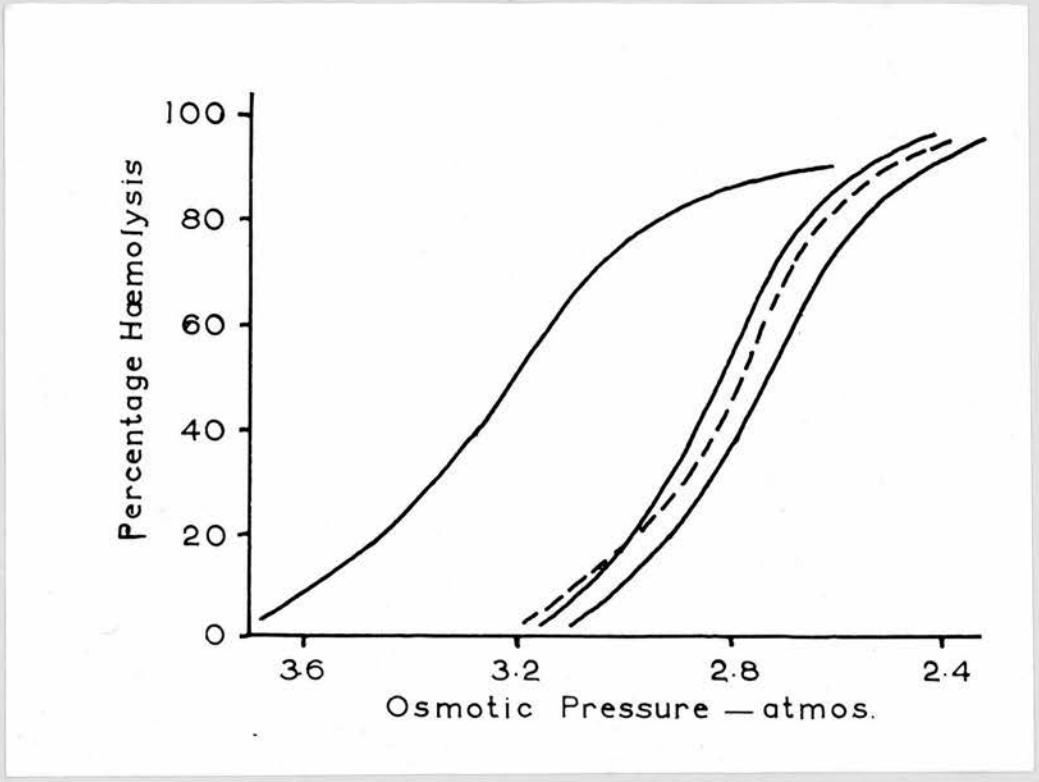

Figure 11. Haemolysis Curves of the Sodium Halides. Reading from L. to R. NaF, NaCl, NaBr (broken line), NaI.

The full significance of these curves is not yet clear; their immediate practical importance lies in the fact that they enable one to interchange cations and anions with predictable results. Only one curve - that of sodium fluoride - is outstandingly different from the others and this must be due to an effect of the fluoride ion. The pH of the system is not an operative factor; numerous determinations of the pH were made on random samples of the mixtures of blood and all ten salts studied. The pH invariably lay between the limits of 7.9 and 8.2. Even in the case of sodium fluoride, at a dilution of 1 in 20, the blood buffers are sufficient to

control the very slight alkalinity of this salt, and to maintain the pH within the above mentioned limits.

According to Wilbrandt (1940) sodium fluoride increases the osmotic resistance of erythrocytes (i.e. decreases the tendency to haemolysis). Unfortunately, Wilbrandt's paper is not accessible, but Hober (1945) quotes Wilbrandt as having recorded an "enormous increase of osmotic resistance" after addition of sodium fluoride to a suspension of human erythrocytes in saline. It was suggested that this fluoride effect was produced by its action in inhibiting glycolysis.

With all five specimens of blood used, haemolysis occurred at a higher osmotic pressure in solutions of sodium fluoride than in solutions of sodium chloride, bromide or iodide. The actual osmotic pressures corresponding to 50% haemolysis (derived from the five specimens of blood used) were:- 3.26, 3.22, 3.20, 3.19 and 3.12 atmospheres. Even at the lowest of these pressures, it is doubtful if one could get even a trace of haemolysis in sodium chloride solution.

At a later stage in this work it became necessary to re-examine the degree of haemolysis in solutions of the alkali halides to determine finally whether the differences between the effects of the various salts were real or due to biological variation of the blood samples which had been used. Certain modifications were introduced. The same

sample of blood was distributed into a series of iso-osmotic solutions of the alkali halides thereby eliminating individual variation from blood to blood, and the percentage haemolysis was determined for each salt. The salts selected were potassium chloride (in which the cells appear to be least resistant), sodium bromide, sodium chloride, potassium bromide and potassium iodide. Another important modification was the reduction of the duration of haemolysis to five minutes; although not of fundamental significance, this shortening of the time of exposure of the cells to the haemolysing system reduces considerably the degree of haemolysis. Two further minor modifications were the use of heparin as anticoagulant in place of oxalate, and a more rigid control of the temperature to within 0.1°C.

Results are given in Table 25 A (p. 78).

Table 25A. The Haemolysis of Erythrocytes in Solutions of the Alkali Halides. Temperature = 20.0°C. Dilution = 1 in 20.

Blood.	Osmotic Pressure. (atmos.)	Percentage Haemolysis. NaBr	KI	NaCl	KBr	KCl
1.	2.82	1	1	1	1	2
2.	2.82	2	4	4	4	6
3.	2.82	1	4	3	4	6
4.	2.82	1	1	2	2	4
5.	2.75	4	7	8	7	14
6.	2.75	10	18	21	18	36
7.	2.75	14	28	33	29	51
8.	2.75	49	70	73	75	84
9.	2.60	57	67	70	70	76
10.	2.60	35	67	57	66	76
11.	2.60	31	57	53	65	76
12.	2.60	34	56	59	55	70

The figures shown in the above Table are not to be taken strictly at their face value. With all such haemolysing systems, the osmotic fragility curve is sigmoid, so that when the percentage haemolysis is in the region of 1-5% or 95-100%, the results are embarrassingly constant for it requires a relatively large change in osmotic pressure in either of these ranges to alter the degree of haemolysis by 1%. Nevertheless, it must be

conceded that haemolysis "begins" at very much the same osmotic pressure in the case of all five salts. Conversely, in the region of 30-70% haemolysis, the systems are maximally sensitive to any alteration in the extracellular conditions, and in this region of the curve, irregularities are more likely to appear.

In the above Table, it is shown that haemolysis in sodium bromide solution is always less than (or equal to) the degree of haemolysis in all the other four salts. The degree of haemolysis in solutions of potassium chloride is always greater than the degree of haemolysis in all the other salts. The degree of haemolysis in sodium chloride and potassium bromide and iodide are intermediate.

In other words, the differences previously observed between the haemolytic actions of the several alkali halides have been confirmed, and they are not due to individual variations in the blood specimens which were used. There is a certain regularity among the effects of the halides of sodium and potassium, for the osmotic pressures in Table 25 corresponding to 50% haemolysis, when re-arranged, are:-

	Sodium	Potassium
Chloride	2.81	3.01
Bromide	2.78	2.88
Iodide	2.72	2.80

But the haemolytic activity cannot be related to

ionic volumes since sodium chloride and potassium iodide have almost identical haemolytic activity, and if ionic volume were a limiting factor, sodium should penetrate the cell (and so produce lysis) more rapidly than potassium whereas, in practice, the cells are more readily haemolysed in the potassium than in the corresponding sodium salt. However, it is by no means certain that penetration by the cation is the fundamental, or even a likely, cause of haemolysis. Cation permeability is known to be a slow process and in the second series of experiments (Table 25A, p. 78) the duration of haemolysis was deliberately reduced to the lowest convenient time of exposure (5 minutes) so that the possibility of any appreciable cation movement across the cell membrane can be ruled out of the possible explanations of the different effects of the various alkali halides. Ponder (1949) has come to the same conclusions.

Whatever the cause of these variations, it must be admitted that it is justifiable to interpret the results in a very different manner. Excluding the case of sodium fluoride, which appears to have an intrinsic haemolytic action, the differences between the osmotic pressures required to produce 50% haemolysis under the conditions of the experiment are very small. The greatest difference from the mean occurs in the case of potassium chloride, and

this difference is only 6.8%, while the differences in the case of sodium chloride (2.81 atmospheres), potassium iodide (2.80), sodium bromide (2.78) and caesium chloride (2.77) are almost negligible.

It would therefore seem reasonable to conclude (a) that the differences in haemolytic action between the alkali halides selected are real and are due to some cause unknown, and (b) that many of these differences are very small - so small that if certain pairs of salts are selected (e.g. sodium chloride and potassium iodide) the difference is virtually nil.

Part 5.

The Effect of Change of Temperature on the Osmotic Haemolysis of Erythrocytes.

It has long been known that a fall in temperature increases the degree of haemolysis in hypo-osmotic sodium chloride solution and in other haemolysing systems, and that a rise in temperature has the opposite effect. Temperature changes have been studied in the range between 0° and 45°C.; beyond either extreme, rapid and complete haemolysis occurs at all concentrations. Several theories have been put forward to explain this effect, and two of these have already been referred to (see pp. 60, 61). In addition, Ponder (1934) has suggested that the increased osmotic resistance at high temperatures is due to the escape of osmotically active substances from the cell interior. Although, as has been shown by many others, potassium can escape quite easily through the cell membrane, the escape is a comparatively slow one - a matter of hours - whereas osmotic haemolysis is extremely rapid - a matter of a few seconds. For this reason, Ponder's view on the matter has met with a good deal of opposition. Lepeschkin (1935) attributes the change in osmotic resistance on cooling, to mechanical damage to the cell membrane; at low temperature the cell is believed to be more prone to injury produced by the swelling which occurs prior to haemolysis. Were this the case, it would be necessary to assume that

the damage is reversible for a volume of cells removed from a vein and cooled to room temperature becomes more resistant when heated to 37°C. again.

There seems to be no reason why this alteration in fragility on changing the temperature should not be explained in terms simpler than any of those theories, more in keeping with classical physical chemistry, and susceptible of practical verification. As the expression implies, osmotic haemolysis depends on the osmotic pressure of the medium in which the erythrocytes are suspended. The osmotic pressure of any solution depends upon (a) the nature of the solute, (b) its concentration, and (c) the temperature. These factors are related in several equations to which reference has already been made (pp. 36, 50).

Alteration in temperature, other things being equal, will have three distinct effects all of which influence the osmotic pressure. These are:-

(1) a contraction in volume on cooling, and vice versa, which will alter the concentration,
(2) in the case of an electrolyte, the degree of ionisation alters with the temperature; within the range 0° - 37° the conductance ratio increases slightly as the temperature increases,
(3) the osmotic pressure is influenced directly - being one of the factors in the van't Hoff equation.

Confining the discussion for the moment to these three factors, it is obvious that as the temperature

falls from 37°, factors (1) and (2) will cause an increase in the osmotic pressure, while factor (3) will produce a decrease. It is now necessary to consider the magnitude of these changes.

(1) When 10.000 ml. of distilled water at 18° is cooled to 0°C. the volume of the liquid is reduced to 9.988 ml. - a decrease of 0.012 ml. (or -0.1%) in volume and there is therefore a corresponding increase of +0.1% in concentration.

(2) At 18°C. the value for the degree of ionisation, calculated from either freezing-point or conductivity data, for M/20 sodium chloride is 0.879, and at 0°, the value rises to 0.888. In the case of a uni-univalent electrolyte the factor introduced into the van't Hoff equation is 1.879 at 18° and 1.888 at 0°C. This change in the value of $(1 + \alpha)$ will make a difference of +0.5% in the osmotic pressure when the temperature falls from 18° to 0°. This variable can be eliminated by the use of a suitable non-electrolyte, but the vast majority of experiments on this point have been carried out with solutions of sodium chloride and it is proposed to continue with the use of this salt.

(3) In the calculation of the osmotic pressure there are several equations in use. Fortunately, when dealing with solutions of molarity of the order of M/20, it makes little difference whether one uses the original van't Hoff equation, the more elaborate

Morse equation, or the ideal thermodynamic equation. Glasstone (1946, p. 671) gives the following figures for the osmotic pressure (in atmospheres) of a solution of sucrose at 30°C. containing 0.1 mole per 1000 g. of water:-

van't Hoff equation (calc.)	2.40
Morse equation (calc.)	2.47
Ideal equation (calc.)	2.44
Observed value	2.47

For the sake of simplicity, the original van't Hoff equation has been used in all the following calculations.

A solution of sodium chloride in which the average blood haemolyses to the extent of 50% (see Table 23, p. 69) contains 0.366 g. sodium chloride per 100 ml. (approx. M/16) and has, according to the van't Hoff equation, an osmotic pressure of 2.795 atmospheres at 18°C. and 2.622 atmospheres at 0°C. (assuming the value of α to be constant at 0.870 for M/16 sodium chloride and ignoring the change in volume at the two temperatures). This represents a change of 0.173 atmospheres, equivalent to a change of -6.2% in osmotic pressure when the temperature falls from 18° to 0°C.

Factors (1) and (2) each produce a small increase in osmotic pressure as the temperature falls, while factor (3) produces a relatively large decrease - the net result being a change of -5.6% in the osmotic pressure.

At this point, it may be stated that the original concept of a degree of ionisation has been considerably modified by the work of Debye and Huckel, and more recently by Onsager, who have introduced and elaborated the theory of complete ionisation in which freedom of movement of the ions is to some extent impeded by their ionic atmospheres. The original degree of ionisation has been replaced by the activity coefficient ($f_{\pm}$). In the following table the activity coefficients quoted are those given by Glasstone (p. 965); the values of α have been calculated from data given in the International Critical Tables.

Sodium Chloride Solutions at 25°C.

Molarity.	0.001	0.005	0.01	0.05	0.10	0.50
$f_{\pm}$	0.97	0.93	0.90	0.82	0.78	0.68
α	0.98	0.95	0.94	0.88	0.84	0.74

The differences between $f_{\pm}$ and α are small but significant, but provided that one adheres to the one or the other, the errors introduced are not likely to affect the conclusions in any important detail. The original degree of ionisation has been used in all the following calculations.

When the temperature is increased from 18°C. to 37°, similar changes will occur, but in the opposite direction. The concentration will fall slightly due to expansion of the solution, the degree of

ionisation will decrease, and the osmotic pressure calculated by the van't Hoff equation will increase. As before, the change due to the third of these factors will outweigh the others, and the net result will be an increase in osmotic pressure. For example, the solution of sodium chloride containing 0.366 g. per 100 ml. will have the following osmotic pressures (ignoring the expansion of the solution, and assuming α to remain constant at 0.870):- at 18°C., Π = 2.779 atmos.; at 37°C., Π = 2.961 atmos. The increase is 0.182 atmos., or +6.5%.

In these circumstances, the osmotic pressure of the solution containing 0.366 g. sodium chloride per 100 ml. will increase by 0.173 + 0.182 = 0.355 atmospheres between 0° and 37°.

The average haemolysis curve of oxygenated blood at a dilution of 1 in 20 and at 20°C. is sigmoid and 10% haemolysis corresponds to a concentration of 0.395 g. NaCl per 100 ml. while 90% haemolysis corresponds to a concentration of 0.332 g. NaCl per 100 ml. These concentrations are equivalent to osmotic pressures of 3.04 and 2.55 respectively in atmospheres at 20°C. (α = 0.870). The difference (0.49 atmospheres) is rather greater than the change in osmotic pressure which would be produced by altering the temperature from 0° to 37°, but is of the same order. The effect of such a change of temperature on the degree of haemolysis at any specified concentration of sodium chloride will

be considerable, and if it can be shown that the observed results are in quantitative agreement with the above discussion, it may be concluded with some degree of confidence that the change in the degree of haemolysis with change of temperature is a function of the accompanying change in the osmotic pressure of the haemolysing system.

Before proceeding to test the theory, there are two further complications to consider. The above arguments have dealt with alterations in the haemolysing system, i.e. the hypo-osmotic solution of sodium chloride to which blood is added. Once the blood has been added, the erythrocytes are now suspended in a mixture of salt solution plus plasma. To this mixture is added the contents of any cells which have haemolysed should the salt concentration have been sufficiently low. In the quantitative treatment of these complications, much depends on the ratio of blood to haemolysing solution. The reasons for selecting a dilution of 1 in 20 have been referred to before.

When normal human blood is used, the final mixture contains 9.50 ml. of haemolysing solution, 0.29 ml. plasma and 0.21 ml. cells (assuming the original blood to have had a packed cell volume of 42% cells). The added plasma which is present does not alter the arguments presented above; its osmotic pressure and absolute volume will also vary with the temperature, and the ionisation of its

electrolytes will depend on the temperature. The quantitative effect of these changes will be the same in magnitude and direction as in the case of the haemolysing solution.

Since normal human blood has been used in all experiments, the effect of the added plasma will be nearly constant: the volume of the added plasma is normally only 1/35th of the total fluid present; but its osmotic pressure is higher than the osmotic pressure of solutions of sodium chloride which will produce haemolysis. The average freezing-point of serum (-0.56°C.) indicates that it has an osmotic pressure at 0° which is the same as that of a solution containing 0.942 g. sodium chloride per 100 ml. Assuming the degree of ionisation to be 0.870, this solution of sodium chloride has an osmotic pressure of 7.19 atmospheres at 18°C., i.e. about 2.5 times the osmotic pressure of a sodium chloride solution which will produce 50% haemolysis.

The addition of 0.29 ml. plasma (Π = 7.19 atmos. at 18°C.) to 9.50 ml. of a solution of sodium chloride containing 0.366 g. per 100 ml. (Π = 2.79 atmos. at 18°C.) will produce a final osmotic pressure of 2.92 atmospheres - neglecting for the moment the effect of the intracellular osmotically active material which will escape from the 50% of cells which have haemolysed. This increase in osmotic pressure is significant, but small. More important is the fact that it is constant in

experiments using normal blood, and in an experiment where a single sample of blood is used throughout, the osmotic effects of the added plasma can be ignored. In experiments in which the blood of different individuals is compared, its neglect will introduce a small error depending on the variation in the packed cell volume of the different blood samples. For the present purposes, it is sufficient to note that the added plasma is affected in the same way as the haemolysing solution as far as temperature changes are concerned.

The second difficulty arises from the increase in osmotic pressure of the haemolysing system due to the escape of osmotically active intracellular material during the course of haemolysis. It has been assumed (although the assumption is not strictly valid) that nothing escapes from the cell until haemolysis begins, and that at the moment of lysis the cell liberates the whole of its contents. In experiments lasting up to half-an-hour, the prelytic escape of material through the cell wall is known to be very small and its effect on the osmotic pressure of the haemolysing system must be minute. In a system sufficiently dilute to allow of haemolysis, the escape of the cell contents must be considered. There will be an increase of this factor in "sigmoid-fashion" as haemolysis approaches completion.

Referring back to the arguments above, before

haemolysis begins, we have 0.21 ml. cells suspended in 9.79 ml. of haemolysing system. Since the normal haemolysis curve approaches 0% and 100% haemolysis asymptotically, it is quite impossible to deal with these levels. But the concentrations corresponding to 10% and 90% haemolysis can be determined with accuracy, and between these limits the haemolysis curve approximates to a straight line (Figure 9, p. 68).

At 10% haemolysis (0.395 g. sodium chloride per 100 ml., Π = 3.02 atmospheres at 18°C.), the osmotic pressure is reinforced by the addition of 0.29 ml. of plasma (Π = 7.19 atmos.) and by 0.02 ml. of intracellular fluid (Π = 7.19 atmos.). It may be calculated that the addition of the plasma raises the osmotic pressure of the haemolysing system from 3.02 to 3.13 atmospheres, and that the addition of 0.02 ml. of intracellular fluid raises it further to 3.15 atmospheres.

At 90% haemolysis, (0.332 g. sodium chloride per 100 ml., Π = 2.54 atmos. at 18°C.) the osmotic pressure of the sodium chloride is reinforced by the addition of 0.29 ml. plasma bringing it up to 2.63 atmospheres, and by the addition of 0.19 ml. of intracellular fluid which raises the osmotic pressure to a final figure of 2.77 atmospheres.

The liberated intracellular fluid thus contributes 0.02 atmospheres at 10% haemolysis and 0.14 atmospheres at 90% haemolysis. These increases

are very small and contribute approximately 0.015 atmosphere for each 10% haemolysis.

The theory is advanced that the change in the degree of haemolysis produced by change in temperature is due to the effect of the change of temperature on the osmotic pressure of the haemolysing system. As the temperature falls, so the osmotic pressure decreases and the degree of haemolysis increases. Between 10% and 90% haemolysis, the haemolysis curve is steep and the difference between these two levels corresponds to a difference of only 0.48 atmospheres at 18°C., so that a very small change in osmotic pressure will bring about a relatively large change in the degree of haemolysis. This change will be most striking at the mid-point of the curve where a change of 0.005 atmosphere (about 1/500th of the total osmotic pressure), brought about by any means, will alter the degree of haemolysis by about 1%. Conversely, below 5% and above 95% haemolysis a large change in osmotic pressure will have a relatively small effect on the degree of haemolysis and these regions of the curve are to be avoided.

In the following experiments, the conditions were those set out on page 66, except that, heparin was used as anticoagulant, the time of haemolysis was reduced to 30 minutes, and temperature was varied in the different experiments from 4° to 35°C.

The following values were used for the degree

of ionisation of the sodium chloride at the temperatures stated:

T°C.	5	10	15	20	25	30	35
α	0.879	0.876	0.873	0.869	0.866	0.862	0.859

These figures were arrived at by extrapolation of values calculated from freezing-point and conductivity data given in the International Critical Tables. They correspond to solutions of sodium chloride which are M/16.0.

Temperature control was effected by water-baths and incubators kept within 0.5° of the desired temperature. All apparatus was equilibrated at the temperature of the solutions which were to come into contact with them. This is most important when working at the higher temperatures - a fall in temperature due to cold apparatus will increase the degree of haemolysis during manipulation. The reverse is unimportant.

Osmotic pressures were calculated from the equation:-

$$\Pi = 0.01403 \cdot (1 + \alpha) \cdot T \cdot c \quad \text{.....(xi)}$$

where c is the concentration of sodium chloride in g. per 100 ml.

Haemolysis at Constant Concentration. An idea of the magnitude of the changes in the degree of haemolysis at different temperatures is shown by the figures in the following Table. The osmotic

pressure at the corresponding temperature has been included for comparison.

Table 26. Haemolysis at Constant Concentration.

Blood A.			
Conc. g.NaCl/100ml.	0.350	0.350	0.350
Temperature (°C.)	4.0	18.0	35.0
II (atmospheres)	2.56	2.67	2.81
Percent Haemolysis	94%	85%	36%
Blood B.			
Conc. g.NaCl/100ml.	0.370	0.370	0.370
Temperature (°C.)	4.5	17.5	35.0
II (atmospheres)	2.71	2.82	2.97
Percent Haemolysis	70%	50%	12%

Haemolysis at Constant Osmotic Pressure. Using the equation on p. 89, it is a simple matter to produce two solutions of sodium chloride which will have the same osmotic pressure at two different specified temperatures. A specimen of blood would then be expected to haemolyse to the same extent in each solution at the corresponding temperature. As might be anticipated, agreement is best towards the two ends of the haemolysis curve.

Table 27. Haemolysis at Constant Osmotic Pressure. Duration of Haemolysis = 30 minutes. Osmotic pressure in atmospheres; concentrations of sodium chloride in g. NaCl per 100 ml.

Blood.	Osmotic Press.	Temp. °C.	Conc. NaCl	Percent. Haem.	Temp. °C.	Conc. NaCl	Percent. Haem.
C.	2.80	11.5	0.3740	8%	20.5	0.3638	7%
D.	2.75	12.0	0.3666	12%	22.0	0.3555	11%
E.	2.60	12.0	0.3466	55%	22.0	0.3361	54%
F.	2.65	5.0	0.3616	10%	20.5	0.3445	11%
G.	2.50	5.0	0.3412	51%	21.0	0.3243	59%
H.	2.60	4.0	0.3561	57%	16.0	0.3423	58%
I.	2.60	18.5	0.3402	45%	34.0	0.3274	39%
J.	2.40	18.5	0.3140	89%	34.0	0.2997	90%
K.	2.60	18.5	0.3402	58%	34.0	0.3274	48%
L.	2.55	4.0	0.3492	39%	35.0	0.3175	32%
M.	2.40	4.0	0.3287	41%	35.0	0.2988	40%
N.	2.55	4.0	0.3492	11%	35.0	0.3175	8%

The average difference between the two sets of figures for the percentage haemolysis is 3%. This difference is so small that there can be no doubt that, at constant osmotic pressure, blood always haemolyses to the same extent whatever the temperature. There are numerous small technical difficulties in experiments such as these. For example, after the system has been allowed to stand at 35°C., it begins to cool down as soon as it is removed from the

incubator, e.g., whilst centrifuging, and, as the temperature falls, the degree of haemolysis increases. Speed of manipulation is therefore vital.

The theory can be checked another way. Three series of solutions, each member differing in concentration from the next by 0.010 g. NaCl per 100 ml., are equilibrated at three different temperatures - in the following example, 6°, 20°, and 34.5°C. A single specimen of oxygenated blood is distributed into each series to a dilution of 1 in 20 and allowed to haemolyse for 30 minutes. The tubes are then quickly centrifuged at 2,500 r.p.m. and the percentage haemolysis is determined in each sample.

When the percentage haemolysis is plotted against the concentration in g. NaCl per 100 ml., three curves are obtained corresponding to the three selected temperatures (see Figure 12, p. 97).

When the percentage haemolysis is plotted against the calculated osmotic pressures in atmospheres, all points lie on a single curve irrespective of the temperature (see Figure 13, p. 97).

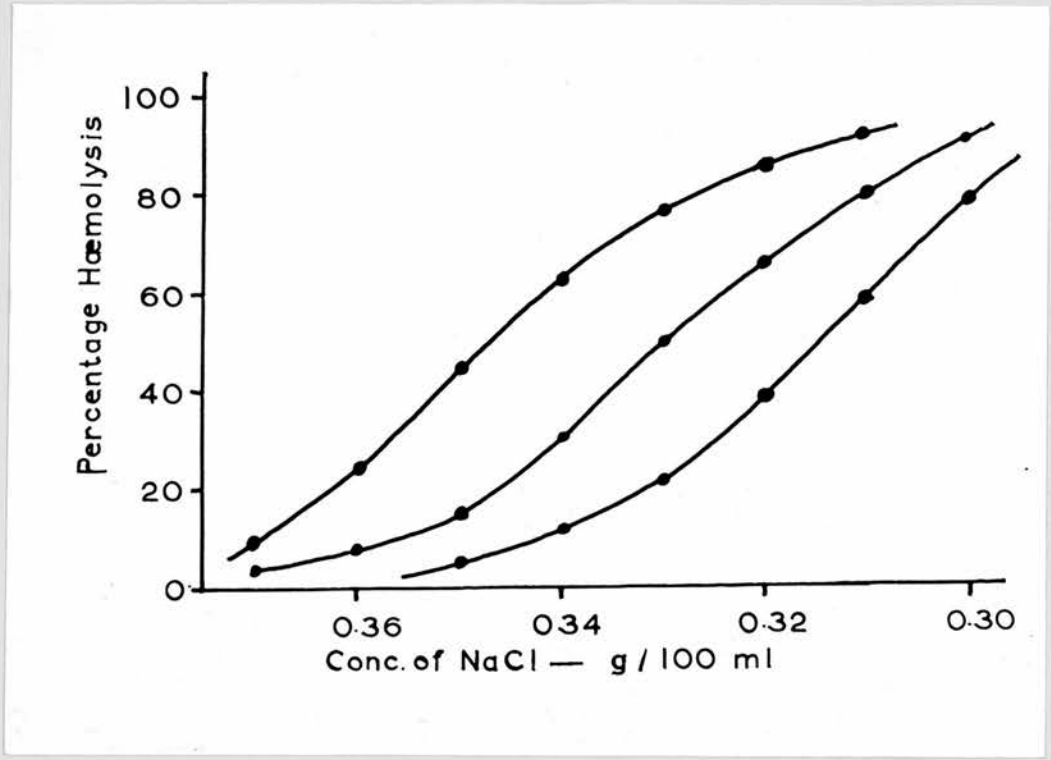

Figure 12. Haemolysis at Constant Concentration.

Left hand curve, 6.0°C. Middle curve, 20.0°C.
Right hand curve, 34.5°C.

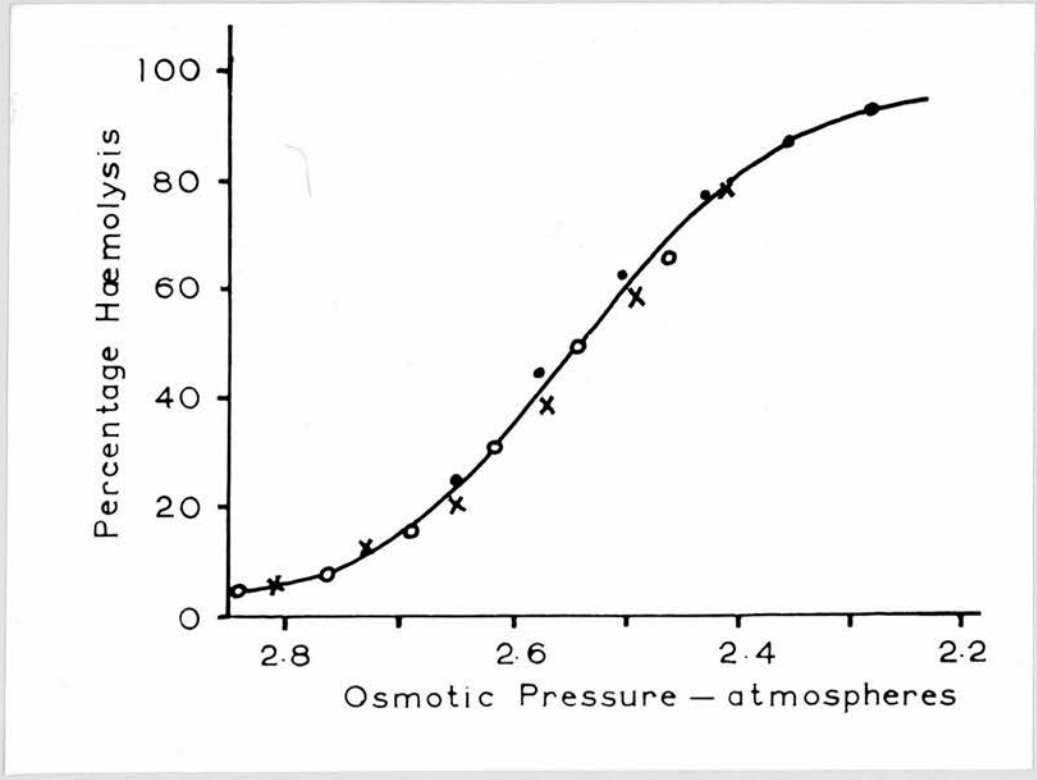

Figure 13. Haemolysis at Constant Osmotic Pressure.

6.0°C. 20.0°C. 34.5°C.

Part 6.

The Red Cell as an Osmometer.

Having examined the effects time factors, pH, electrolyte in the external medium, and temperature, we now turn to an important matter in this series of investigations - the question of whether or not the red cell is a perfect osmometer. *A priori* it might be taken for granted that the red cell membrane is a perfect semi-permeable membrane, yet there is a considerable literature which, with rare exceptions, leads to the conclusion that the red cell is *not* a perfect osmometer and that departures from the ideal behaviour are considerable. But in all dissertations on the subject, one cannot fail to observe that there is an underlying doubt about the conclusions which have been reached. Although seldom said in so many words, the general feeling seems to be that the red cell is, in fact, a perfect osmometer; that the experimental findings are either wrong or are wrongly interpreted; and that none of the several explanations of the red cell's anomalous behaviour are feasible. Certainly, before it is finally accepted that the red cell does not behave as a perfect osmometer, unequivocal proof is necessary and the literature does not contain such proof.

Osmotic haemolysis, such as has been described in the previous sections, is the end-point of osmotic

swelling and whatever may be the precise factors controlling the actual escape of haemoglobin (and they may not be as complicated as some would have us believe) there is no question that the cells gradually swell as the external tonicity decreases until they reach the point at which haemolysis begins. It is the extent of this prelytic swelling which is in doubt. If the cell is to behave as a perfect osmometer, the degree of swelling at any tonicity may be calculated from one or other of two equations. These are:-

$$V = W \left(\frac{1}{T} - 1 \right) + 1 \quad \text{.....(xii)}$$

when the volume of the medium surrounding the cell is infinitely large compared with the volume of the intracellular water, and,

$$V = W \left(\frac{p - pT}{pT + 1} \right) + 1 \quad \text{.....(xiii)}$$

when the volume of the extracellular medium is relatively small compared with the volume of the intracellular water.

In these two equations the initial volume of the cell is taken as unity,
V is the final volume of the cell at equilibrium,
T is the tonicity of the extracellular medium before cell swelling has had time to occur. The tonicity of the plasma is taken as unity,
p is the ratio of the initial volume of the extracellular medium to the initial volume of the

intracellular water, and,
W is the fraction of water inside the cell - commonly taken as 0.70.

These equations are derived on the assumption that the van't Hoff equation holds, i.e., that the cell is a perfect osmometer, and they are mathematically correct.

As has been said before, the experimental and calculated values for the final equilibrium volume of the cell do not agree, and in order to reconcile practice and theory, Ponder has introduced a constant R on the right hand side of both equations in order to correct for the anomalous degree of swelling. The equations then become,

$$V = RW \left(\frac{1}{T} - 1 \right) + 1 \quad \text{.....(xiv)}$$

and,

$$V = RW \left(\frac{p - pT}{pT + 1} \right) + 1 \quad \text{.....(xv)}$$

It will be noted that the factor R governs the degree of swelling, not the final equilibrium volume. If the cell behaves as a perfect osmometer, the observed value of R will be unity.

In practice, an observed value of R equal to unity is rarely found. Very occasionally, the degree of cell swelling is greater than the calculated value (R greater than unity); but in the overwhelming majority of cases, the degree of cell swelling is less than the calculated value.

In the following Table (selected from figures quoted by Ponder (1948)) are given the values of R which have been obtained by haematocrit methods.

Table 28. Values of R which have been obtained by Haematocrit Methods.

Authors.	Animal.	R values.
Ponder & Saslow (1931)	(1) Rabbit	0.58-0.73
Ponder & Robinson (1934)	(1) Rabbit	0.71-0.98
Christensen & Warburg (1929)	(2) Man	0.8
Schoidt (1932)	(2) Man	0.5
Parpart & Schull (1935)	(3) Beef	0.58-0.81
do.	(3) Rabbit	0.58-0.91
do.	(3) Pig	0.67-0.8
do.	(3) Dog	0.6-0.73
Castle & Daland (1937)	(1) Man	0.75
Ege (1921)	(4,5) Rabbit	0.67-0.88
Krevinsky (1930)	(4) Man	0.6
Guest & Wing (1942)	(4) Man	0.95-1.0
Guest (1948)	(4) Man	0.5-1.0

The numbers in brackets refer to the nature of the hypotonic medium in which the cell swelling was measured; (1) plasma/serum, (2) oxalate-phosphate buffer, (3) Ringer-Locke solution, (4) sodium chloride, and (5) glucose.

There are two strong arguments against the use of this constant R. In the first place, the value of R determined experimentally is variable, as the

figures quoted in Table 28 show. In the second place, this constant R has no interpretation other than that of an expression of the amount of deviation of the observed from the calculated. All endeavours to explain the constant R merely add to the confusion. It is therefore quite impossible to accept this factor as having either physical or biological significance, and the whole problem requires a most critical examination.

Before proceeding to a study of cell swelling, two points which have already been discussed in detail, have some bearing on the problem. It has already been shown (Part 5) that although the degree of haemolysis varies with the temperature at constant concentration, it is independent of the temperature at constant osmotic pressure. Proof of this fact entailed the use of the van't Hoff equation - it assumed that the red cell was a perfect osmometer reacting in a predictable manner when the extracellular osmotic pressure was altered. It has also been shown (Part 4) that red cells haemolyse almost (but not quite) to the same extent in solutions of a variety of alkali halides. Admittedly, differences have been shown by two series of experiments, but these differences are small and when certain pairs of salts are selected, the differences are virtually zero.

These observations show that the red cell behaves as a perfect osmometer under conditions of varying

temperature, and as an almost perfect osmometer under conditions of varying alkalihalide in the extracellular fluid. It would therefore be somewhat unexpected if it could be shown that the red cell was a very imperfect osmometer under conditions of varying external tonicity.

As a preliminary investigation, the value of R was determined by haematocrit methods in a series of 34 experiments on 8 different samples of blood. Two variations of the accepted method of determination were used; (1) heparinised, oxygenated blood was accurately diluted with either 2.00 or 3.00 volumes of hypotonic sodium chloride solution, and the packed cell volumes of the mixtures were determined in straight Hawksley 100 mm. haematocrit tubes. (2) the blood was diluted 1 in 48 with hypotonic sodium chloride solution in Van Allan haematocrit tubes (These are tubes with a graduated capillary stem surmounted by a bulb, so that relatively high dilutions are obtained and yet the packed cell volume may be accurately measured). In both types of experiment, the blood was centrifuged at 3,000 r.p.m. for 30 minutes.

It is unnecessary to give further details of the experiments or to tabulate the protocols. The experiments followed the usual pattern - so did the results. In the 34 experiments, the values of R lay within the limits of 0.67 to 0.79 with an average of 0.74. This figure stands comparison with most

of the results quoted in Table 28 and leads to the conclusion that the red cell is not a perfect osmometer.

The possible causes of this will now be investigated seriatim.

Certain possible sources of error are not easily susceptible of investigation. Even in stating the problem, it is assumed that the alkali halides do not penetrate the cell and that osmotically active material neither enters nor leaves the cell during the determination of the cell volume. It has long been known and repeatedly confirmed, that when cells are suspended in hypotonic saline, potassium leaves the cell. But all observers are agreed that this escape of potassium is very slow whereas the swelling of the erythrocyte when placed in hypotonic saline is all but instantaneous. No correction for loss of osmotically active material will raise the value of R to unity (Ponder (1943-44)).

Were further evidence required, the following simple experiment could be added.

If potassium leaves the cell and nothing enters to replace it (i.e., the conditions required by the low value of R) then water will also leave the cell in order to maintain osmotic equilibrium, and the cell volume should therefore decrease when cells are allowed to stand in contact with hypotonic saline. In practice, they do not. The cell volume increases

by about 1% per hour at 37°C. This increase does not contradict the fact that potassium leaves the cell. It certainly does. But some other ion (sodium, presumably) enters in an amount osmotically slightly greater than the potassium which leaves. There may be other explanations, of course, but such an experiment is further proof that the rapid anomalously small swelling of the red cell is not due to the very slow transfer of electrolyte across its membrane.

Heparin, in minimal amount has been used as the anticoagulant. There is no evidence that a trace of heparin has any effect either on cell swelling or on the degree of haemolysis, and certainly, of all methods of preventing clotting, it is the least objectionable. Whole blood has been used in all experiments in preference to washed cells, partly to avoid delay and partly to avoid any possibility of damage to the cells by repeated washing and centrifuging. It is a simple matter to calculate the tonicity of the extracellular fluid if the tonicity of the plasma is taken as unity.

As in studying the degree of haemolysis, so in determining cell volumes, the blood must first be oxygenated to eliminate changes in volume during manipulation due to the loss of carbon dioxide. Table 29 shows that oxygenation alone produces an appreciable decrease in cell volume.

Table 29. The Effect of Oxygenation on the Packed Cell Volume.

	Packed Cell Volume. %age Cells.		Haemoglobin. g. per 100 ml.	
Sample.	Venous.	Oxygenated.	Venous.	Oxygenated.
A	40.0	38.3	13.8	13.8
B	45.6	43.8	14.4	14.4
C	45.9	43.8	14.8	14.8
D	45.8	43.3	14.6	14.7
E	50.3	48.1	16.4	16.3
F	42.2	40.1	14.1	14.0

These determinations were made by centrifuging at 3,000 r.p.m. for 30 minutes. The average decrease in packed cell volume due to oxygenation is 4.5% of the initial volume. The constancy of the haemoglobin concentration excludes the possibility of the blood having been diluted by taking up water from the moist oxygen (the oxygen used was first bubbled through water to prevent evaporation of the plasma during oxygenation). This decrease in cell volume due to oxygenation corresponds to the decreased fragility of oxygenated blood. Failure to oxygenate at the start of the experiment may account partly for the irregularity in the values of R, but such an omission is not a major source of error for the values of R obtained with venous blood are no worse than they are with oxygenated blood. Nor can such an omission be the cause of the low values of R

obtained when washed cells are used, for the process of washing must bring about a fairly complete oxygenation.

The accepted value of 0.70 for the fraction of water in the cell (W in equations xii to xv) comes under suspicion. If this value could legitimately be reduced to 0.52, the value of R would automatically rise from 0.74 to unity. The water content of red cells is usually determined by drying a mass of packed cells to constant weight at 60-80°C., and the possibility exists that the accepted value of 0.70 might include some water of constitution driven off by partial decomposition at these temperatures.

The following straightforward experiments effectively ruled out this idea.

Oxygenated, heparinised blood was centrifuged in very narrow tubes to ensure complete packing at 3,500 r.p.m. for 45 minutes. After removal of the plasma, the white cell layer, and the top of the mass of packed cells, the lower part of the red cell layer was taken up by pipette, transferred to a silica boat, weighed, and dried to constant weight over calcium chloride at room temperature and atmospheric pressure. The dried mass was then transferred to a special all-glass drier (Quickfit and Quartz) and further dried over concentrated sulphuric acid at 60°C. at atmospheric pressure until constant weight was again reached. The density of the red cell was taken as 1.099. The results were:

Blood Specimen.	First drying. Fraction of Water.	Second drying. Fraction of Water.
A	0.690	0.697
B	0.695	0.701
C	0.691	0.700
Means.....	0.692	..... 0.699.

The existence in the erythrocyte of water which is not osmotically active ("bound" water) would, were it present in sufficient amount, have the effect of eliminating the factor R. Neither the presence nor the absence of bound water in the red cell has been proved or disproved, but the concensus of opinion is that the amount of bound water in erythrocytes is very small, if it exists at all - see Hill (1930), Macleod and Ponder (1936) and Blanchard (1940). But even if bound water in the erythrocyte were a factor in this problem, it might explain the low values of R, but it could not explain the variations in the experimental values of R. As Ponder (1943-44) says, the several values of R recorded by different investigators would require that the bound water vary between 50% and 100% of the total water in the cell.

Mechanical damage to the cell membrane is not believed to be an important factor since dilute suspensions (1 in 20) of normal red cells in moderately hypotonic saline subjected to mechanical shaking for some hours do not show haemolysis, and

furthermore, they can be subjected to centrifugal forces several thousand times that of gravity without rupture of the membrane provided that the extracellular osmotic pressure is above a certain limit (about 3.5 atmospheres). Parpart and Ballantine (1943) have shown that the cells of whole blood suspended in their own plasma can be subjected to a centrifugal force of 30,000 x g without lysis, and that they can be re-suspended and re-packed several times without alteration of the packed cell volume.

Apart from mechanical damage, the cell membrane may be able to prevent or limit changes in shape or volume by means of its elasticity, and so prevent the theoretical degree of swelling being reached. The degree of osmotic swelling which can be attained without stretching the cell membrane, i.e., the increase in volume occurring as a result of the disc-sphere transformation, is obscured by the difficulty of determining the surface area of a biconcave disc. According to the calculations of Ponder (1949-50) the red cell can increase its volume 2.25 times by changing from a disc to a sphere of the same surface area; according to Guest and Wing (1942) and Guest (1948) the figure is only about 1.65 times. This point cannot be settled until we have some accurate method of determining mean surface areas, but experience tends to confirm the figure of Guest as a first approximation since anything over a 70%

increase in volume results in haemolysis, at least in dilute sodium chloride solutions. Whatever be the correct value, it is quite certain that the red cell can increase in volume (by the disc-sphere transformation) without change in surface area; that is, without any stretching of the cell membrane so that elastic forces are unlikely to be a factor in preventing or limiting osmotic swelling. Ponder (1943-44) favoured the view that cell swelling was opposed by the elastic properties of the cell membrane, and that R was an indirect measure of the opposing force. The only reasonable source of such a force would be an internal structure joining the concavities of the cell and such a structure has never been shown to exist. And once again, while such a theory would account for the low average values of R, it would not account for the variations in the observed values of R.

It is very doubtful if molecular dissociation in the surrounding medium could be a factor. Such dissociation could occur by dilution of the plasma with water (e.g., an increase in the degree of ionisation by dilution) but the effects of such a change would be very small. To produce an anomalously small degree of swelling, molecular association would have to occur within the cell, and such a possibility, when the intracellular contents are being diluted with water, is virtually impossible.

Alteration in pH is known to have a marked effect both on osmotic haemolysis and osmotic swelling, but when oxygenated blood is used, changes in pH are reduced to a minimum. In several experiments, the maximal change in pH which has been found during the sum total of the manipulations involved, never exceeded 0.04 unit and was generally of the order of 0.02 unit. This is quite insufficient to account for the low values of R.

Ponder (1950) has recently suggested that haemolysis may occur in a step-wise manner and that such a process may account for the anomalously small swelling of red cells in partially haemolysed systems. The proposition is not at all attractive, and even if it were the case, it would still not explain why the cell appears to behave as an imperfect osmometer in unhaemolysed systems. He has also suggested (1950-51) that the presence of semi-rigid ghost cells may affect the tonicity-volume relations, but such interference is possible only in partially lysed systems with which we are not concerned.

These various minor possibilities have been mentioned in order to show that many attempts have been made to explain the low experimental values of R. The low values of R are accepted and every endeavour has been made to explain them. But there is one remaining source of error which has yet to be examined - the haematocrit method of determining

relative cell volumes. If one believes that the erythrocyte is a perfect osmometer, then the cause of the lack of agreement between experiment and theory must, by a process of exclusion, lie in the haematocrit determinations.

The first determinations of the packed cell volume were made by Hedin about 1891 and for the last fifty years, this determination has become increasingly popular - especially in the last ten years or so when the errors involved in red cell counting began to be appreciated. It is generally agreed that packing is efficient after 30 minutes' centrifuging at a centrifugal force of 2,000 x g, or more. That a certain amount of plasma remains trapped in the red cell mass, has never been denied, and most recent investigations on the accuracy of this determination revolve around estimations of the "trapped plasma". Vazquez, Newerly, Yalow and Berson (1952), Chaplin and Mollison (1952), and others, are agreed that under these conditions, the trapped plasma constitutes less than 2% of the packed cell volume. There is also evidence that red cells, suspended in their own plasma, undergo compression only at extremely high speeds of rotation (Parpart and Ballantine (1943)). It is therefore reasonable to conclude that the haematocrit method of determination of the packed cell volume of whole blood is sufficiently accurate for the purpose. If

the cause of the low values obtained for R lies with the haematocrit, the error must occur in the determination of the volume of the swollen red cells.

This has been found to be the case, and the key to the remainder of the work in this section lies in the appreciation of the difference between determining the packed cell volume of normal, biconcave, erythrocytes and determining the packed cell volume of swollen, spherical, erythrocytes.

It is a fact that the assumption has been made that since the packed cell volume of normal erythrocytes can be accurately determined by the haematocrit, the same method can be applied to artificially swollen erythrocytes. This is not so. The possibility that the error may arise at this point does not seem to have been considered. Ponder (1949) comes near to the solution in a footnote which concludes:-

"I have not been able to obtain the high values
"of R found by Guest for human cells in hypotonic
"NaCl, except at relatively low rates of spinning.
"Recently I had the experience of obtaining, in
"a succession of determinations, R values between
"0.85 and 0.95 instead of the usual 0.7 to 0.8
"(systems of human red cells in hypotonic NaCl).
"This was traced to the haematocrit motor
"needing oiling."

Criticism of the published conclusions on red cell swelling are complicated by the absence of information on the relative centrifugal force used

in the experiments. The conditions of centrifuging are almost invariably expressed in terms of rotational speed and time from which the relative centrifugal force cannot be calculated unless the effective radius of gyration is known. Furthermore, there are considerable differences between the conditions of centrifuging used by different investigators. Ponder (1948, p. 56) gives illuminating examples of this point. And finally, it is just such variations that are found in the value of R.

The whole matter can be put to the test in an experiment of the following type.

The packed cell volume of a specimen of oxygenated heparinised whole blood was determined by centrifuging at 2,000 x g for 30 minutes in a sealed haematocrit tube. The value found was 45.8% cells, and this value was assumed to be correct, i.e., no correction was made for plasma trapped in the cell mass.

1.00 volume of the blood was added to 2.00 volumes of hypotonic sodium chloride solution and the mixture was stored at 20°C. during the experiment. After thorough mixing, samples were withdrawn, transferred to 100 mm. haematocrit tubes, and centrifuged under various relative centrifugal forces for 30 minutes. Two identical centrifuges were used so that estimations could be carried out every 15 minutes.

If the cells in this system had not altered in volume, the packed cell volume of the mixture would have been 1/3 of 45.8 = 15.3% cells. The hypotonic solution of sodium chloride was such that the relative cell volume at equilibrium (calculated from equation xiii) was 1.45 times the initial volume when W was taken as 0.70. The initial cell volume is taken as unity.

The results are given in Table 30.

It may be repeated that the factor R governs only the swelling of the cell. Thus if the calculated equilibrium volume of the cell is 1.45 and the observed equilibrium volume of the cell is 1.37, then R = 37/45 = 0.82.

Table 30. The Dependence of R on the Centrifugal Force.

Centrifugal Force. (x g.)	Percentage Cells (observed).	Relative Cell Volume.	R.
150	27.0	1.76	1.69
375	23.7	1.55	1.22
500	23.3	1.52	1.15
890	21.9	1.43	0.96
1285	21.1	1.38	0.84
2440	21.0	1.37	0.82

When the centrifugal force is plotted against R, a smooth curve is obtained, and a value of R equal

to unity is obtained only under a centrifugal force of about 750 x g applied for 30 minutes. Duration of centrifuging is as important as the relative centrifugal force and the value of R can be substantially increased or decreased by reducing or prolonging, respectively, the time of centrifugation.

The determinations of R by the haematocrit which were made in a preliminary study (see p. 103) were carried out at a relative centrifugal force of about 3,000 x g - hence the low average value which was obtained (0.74).

There can be no doubt that the high values of R obtained at low centrifugal forces (750 x g or less) are due to the presence of trapped plasma in the cell mass, for when whole blood is centrifuged at 2,000 x g some 2% of plasma remains in the red cell layer. No special significance can be attached to a relative centrifugal force of 750 x g applied for 30 minutes or any other combination of centrifugal force and time which will give a value of R equal to unity. And few would cavil at the conclusion that the low values of R obtained at high centrifugal forces (750 x g or more) are due to compression of the swollen cells. There is no other possible explanation. A value of R equal to unity, obtained for centrifuging at 750 x g for 30 minutes, is the result of a balancing of the errors of trapped plasma in the cell mass and cell compression.

Whatever the magnitude of the errors introduced by trapped plasma and cell compression, the fact remains that a value of R equal to unity can always be obtained by a little judicious manipulation of the centrifuge controls. The conclusion then reached is that all estimations of R which depend on high-speed centrifuging of swollen erythrocytes are valueless, but it still remains to be proved, by acceptable methods, that the red cell is a perfect osmometer. Such methods must avoid high-speed centrifuging.

Several alternative methods have been used in the past with varying degrees of success. Of these, the most attractive is the method of G. N. Stewart (1899) which is based on the principle that when the cell swells by imbibing water, the concentration of any non-penetrating solute in the extracellular fluid will alter by an amount proportional to the amount of fluid which enters the cell. Stewart himself used the animal's own haemoglobin for the reason that he would be quite sure that it would not cross the cell membrane. It has the great disadvantage, however, that any haemolysis in the system will be effectively disguised. It is also a difficult matter to prepare and preserve solutions of pure haemoglobin entirely free from smaller molecules and ions which will interfere with the extracellular osmotic pressure. Shohl and Hunter (1941) used Evan's blue as the extracellular

"indicator", but this also has the effect of masking any haemolysis which may occur. It has other disadvantages as well. Commercial samples of the dye contain a good deal of chloride which is a most objectionable contaminant in experiments dealing with osmotic swelling, but which can be removed by dialysis. More serious is the fact that the extinction of solutions of Evan's blue are altered by the addition of sodium chloride. Thus, starting in each case with 1.00 ml. of a solution of purified Evan's blue:-

Volume of Diluent.	Extinction.	Decrease.
9.00 ml. water.	0.512	-
9.00 ml. 0.5% NaCl	0.476	7%
9.00 ml. 1.0% NaCl	0.461	10%
9.00 ml. 1.5% NaCl	0.450	12%

Ponder (1943-44) also used the Evan's blue method of determining relative cell volumes in vitro and in four experiments using heparin as the anti-coagulant, obtained values of R ranging from 0.97 to 0.93 (mean 0.95). But in 12 experiments when oxalate was used as the anticoagulant, the values of R lay between 0.97 and 0.52 (mean 0.73).

A few preliminary experiments with Evan's blue were not encouraging, and it was finally decided that the best "indicator" of changes in cell volume was the changes in the concentration of total extra-cellular protein. It has the following advantages:-

(1) no extraneous matter (naturally-occurring or otherwise) is added to the system,

(2) it is quite certain that protein does not cross the cell membrane in either direction in measurable amounts,

(3) should any haemolysis inadvertantly occur, it is immediately obvious,

(4) the protein concentration is independent of the concentration of any other constituent, e.g., chloride, and,

(5) the biuret method of estimating protein is highly reproducible, and the technique is such that multiple analyses can be carried out very conveniently, thus reducing the unavoidable errors.

Against these advantages can be cited:-

(1) that small errors in the determination of the protein concentration will lead to greatly magnified errors in the value of R. (This difficulty is common to all procedures of this type, but is overcome by taking the mean of a number of replicate analyses, thereby going far to eliminate incidental errors),

(2) that centrifuging is still necessary, but since its only object is to provide samples of supernatant fluid for analysis, the duration and applied centrifugal force can be reduced to a minimum,

(3) that dilution of plasma may precipitate the more

insoluble globulins. This danger is more theoretical than real. In the experiments to be described, the plasma is not sufficiently diluted for this to be a source of error, and the relatively high pH of oxygenated blood will help to keep the globulins in solution.

Since everything revolves around the accuracy of the determination of small differences in protein concentrations, it is necessary to examine the accuracy of the biuret reaction critically. The most important points for the present purposes are:

(a) the method must be standardised by Kjeldahl nitrogen analyses using the conventional factor 6.25 to convert protein nitrogen to protein. In this case, neither the standardisation nor the accuracy of the conversion factor are of any great moment, for all that is required of the method is that it shall give accurate comparative readings on three protein-containing fluids all derived from the same plasma. For the same reason, the ratio albumin/globulin/fibrinogen in the plasma is of no importance. There is no correction for non-protein nitrogen.

(b) all authors who have used the method in any of its modifications mention the high degree of reproducibility which can be obtained by it, (see Fine (1935), Kingsley (1939, 1942), Robinson and Hogden (1940), Gornall, Bardawill

and David (1949) and others). This is the great advantage of the method in the present experiments. Thus, for example, when 8 analyses were carried out on the same specimen of plasma the measured extinctions were:- 0.318, 0.318, 0.320, 0.320, 0.320, 0.320, 0.320, 0.321 (mean = 0.3196), and the probable error in the analysis of any sample must be considerably less than 1%. The modification of Fine (1935) was used and the simplicity of the method makes it particularly suitable for the duplication of analyses. Exactly the same conditions (time intervals, reagents, etc.) were used throughout the entire series of analyses.

(c) over the range of extinctions used, Beer's law is obeyed.

Before proceeding with the determination of R, it is necessary to decide what method shall be used to determine the percentage volume of cells in the original specimens of blood. Two methods are available, (1) the determination of the packed cell volume by the haematocrit, and (2) the determination of the percentage cell volume by means of protein analyses. Since haematocrit methods are under suspicion, the latter method was selected, but both were actually carried out and the results will be compared later. In order to determine the

percentage cell volume by means of protein analyses, it is first necessary to determine that concentration of sodium chloride which is isoplethechontic with plasma (i.e., that sodium chloride concentration which does not alter the volume of the erythrocytes).

Isoplethechontic Sodium Chloride.

The method used was as follows:-

The packed cell volume of heparinised, oxygenated blood is determined in duplicate by centrifuging in 100 mm. haematocrit tubes (sealed with rubber caps) at 2,000 x g for 30 minutes. A small technical point here is that, with some centrifuges, centrifuging at this speed produces a marked rise in temperature; such centrifuges are unsuitable for this type of work.

Five reference solutions of sodium chloride are prepared containing 1.000, 0.950, 0.900, 0.850 and 0.800 g. per 100 ml. To 2.00 ml. of each solution is added 2.00 ml. of the blood; the systems are thoroughly mixed, allowed to stand at 20°C. for a few minutes, and the packed cell volume determined in duplicate under the conditions described above. Note that it is permissable to subject such blood-saline systems to high-speed centrifugation, for even when mixed with the most hypotonic solution of the above series, very little cell swelling occurs and the cells still retain the form of a biconcave disc.

The five observed packed cell volumes are plotted against the corresponding concentrations of sodium chloride and a graph is drawn through the points. That concentration of sodium chloride which corresponds to one-half of the packed cell volume of the original blood is taken to be the concentration which is isoplethechontic with the plasma of that blood. For example, in one sample of blood, the mean packed cell volume of the whole blood was 40.95% cells. The graph obtained was:-

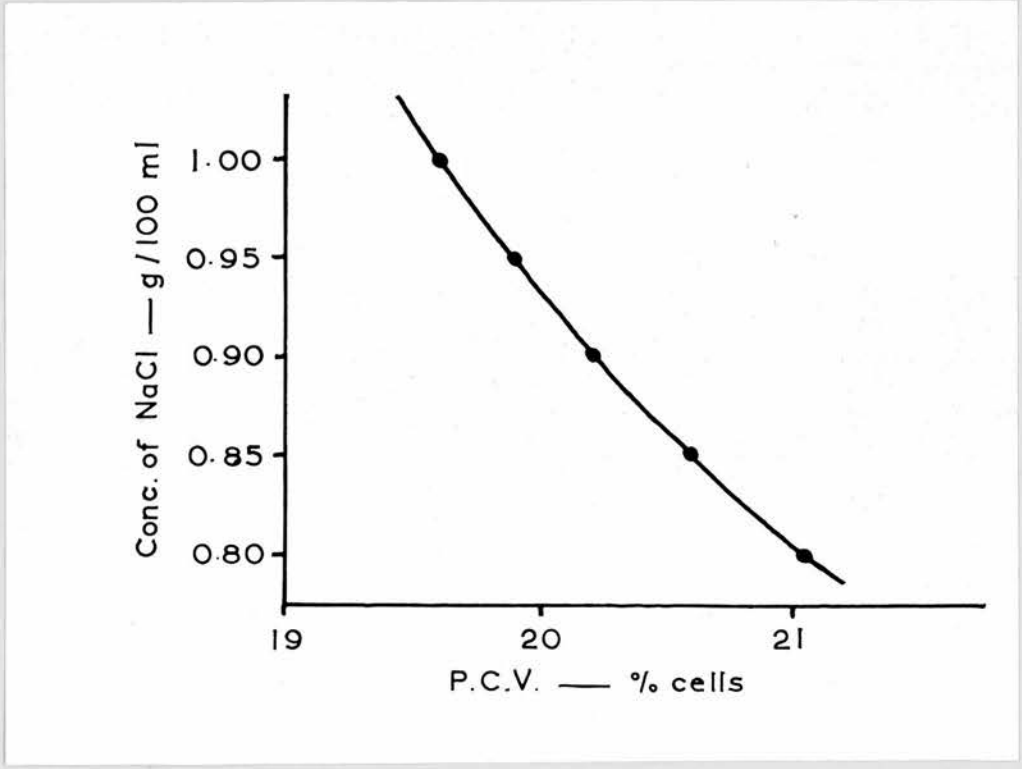

Figure 14. The relationship between the observed packed cell volume and the concentration of sodium chloride.

From this graph, the concentration of sodium chloride corresponding to a volume of 20.5% cells

is 0.862 g. per 100 ml.

Ten such determinations were made on the blood from ten different normal individuals. The blood specimens were all collected between 10-10.30 a.m. and none of the subjects were either dehydrated or oedematous. The values found were:-

0.866	0.875	0.848	0.895	0.880
0.862	0.874	0.885	0.860	0.870

Range: 0.848 to 0.895,

Mean : 0.871 g. NaCl per 100 ml.

It is difficult to say whether this range of normal variation is greater or less than one would expect since the degree of normal hydration in humans is even more difficult to define. The mean is about half-way between the two commonly quoted concentrations for "normal saline" but much of the bicarbonate in the original blood specimens had been removed by oxygenation.

As a matter of interest, one further specimen of blood was collected from a patient who was moderately dehydrated. This was a case of hypertensive encephalopathy who had been unconscious for 24 hours and who died 3 days later. The concentration of sodium chloride found to be isoplethechontic with the plasma was 0.972 g. per 100 ml.

In the experiments leading directly to the determination of R, a concentration of 0.871 g. sodium chloride per 100 ml. was taken to be

isoplethechontic with all plasmas. Ideally, this concentration should be determined for each plasma but this would mean that very large amounts of blood (about 25 ml.) would be required for each experiment, and that the start of the experiments to determine R would be considerably delayed. It was felt that the errors involved in working with blood which was old might well be greater than the error involved in accepting the above solution as standard.

The Determination of R.

This involves three protein analyses, (1) on plasma, and on the supernatants obtained from, (2) a mixture of the blood with isoplethechontic sodium chloride, and (3) a mixture of the blood with water.

About 10 ml. of heparinised blood is fully oxygenated. (As a side-experiment, the packed cell volume was also determined in duplicate by centrifuging in 100 mm. capped haematocrit tubes at 2,200 x g for 30 minutes). The procedure is then:-

(a) Two ml. of the blood is centrifuged at 3000 r.p.m. for 10 minutes and the plasma is removed by a clean dry pipette for analysis.

(b) To 3.00 ml. of the blood is added 2.00 ml. of isoplethechontic sodium chloride. After mixing, the tube is centrifuged at 2000 r.p.m. for 2 minutes and the plasma-saline supernatant is removed for analysis.

(c) 3.00 ml. of the blood is placed in a test tube

which is then centrifuged at 2000 r.p.m. for 5 minutes. When removed from the centrifuge, care is taken not to disturb the cells which are only loosely packed. (Up to this point, all centrifuging has involved only normal, biconcave, cells so that the speeds and durationsof centrifuging are not critical). To the plasma is added 2.00 ml. of distilled water which must be added without disturbing the cells. The thin layer of white cells goes a long way to protect the erythrocytes. The water is carefully mixed with the plasma and the diluted plasma is then rapidly mixed with the cells. After allowing the system to stand for a few minutes with occasional gentle mixing, the tube is centrifuged for exactly 2 minutes at a centrifugal force not exceeding 500 x g. The plasma-water supernatant is then removed for analysis.

Should any haemolysis have occurred in the blood-water system, the whole experiment must be abandoned. In practice, no haemolysis was found in any of the experiments: the extent of cell swelling under these conditions is about 53-54% which is much less than the maximum amount of swelling that the red cell membrane can tolerate (i.e., about 65-70%). Seven experiments in all were carried out. One had to be abandoned because the protein in all three supernatants refused to precipitate completely with trichloracetic acid. The cause of this was never discovered. The

experiments were carried out at 18 ± 1°C. The protein analyses were carried out in quadruplicate and the mean value taken. If any analysis varied more than 1% from the mean, that analysis was discarded, and a fifth estimation carried out.

The Percentage Cell Volume.

This is calculated from the protein analysis of the plasma and the plasma-saline supernatant on the assumption that no water moves across the cell membrane and that the membrane is impermeable to protein. Thus, in the second experiment, (see Table 32, p. 130)

Plasma .....72.0 mg. protein/ml.

Plasma-saline supernatant...33.1 mg. protein/ml.

Let x = the volume of plasma in 1 ml. of blood.

In 3x ml. plasma there is 3x . 72.0 mg. protein,

In 3x + 2 ml. plasma-saline supernatant there is

(3x + 2) . 33.1 mg. protein.

Since no protein crosses the cell membrane,

$3x \cdot 72.0 = (3x + 2) \cdot 33.1$

$x = 0.567$ ml. plasma per ml. blood,

Hence 0.433 ml. cells per ml. blood,

Percentage cell volume = 43.3% cells.

The percentage cell volume found by this calculation is then used in the determination of R.

At this stage, we may digress to compare the packed cell volumes obtained by the haematocrit method with the percentage cell volumes calculated

from protein analysis.

Since it is known (see p. 112) that the packed cell volumes obtained by haematocrit methods contain about 1-2% of plasma trapped in the cell mass, one would expect that the haematocrit values would be consistently 0.4 - 0.8% (of cells) greater than the values calculated from protein analyses of normal bloods which contain about 40% cells. This constant difference was not found.

Table 31. Comparison of the Methods of determining Percentage Cell Volume.

Expt. No.	(1) By Protein Analysis. % cells.	(2) By the Haematocrit. % cells.	Difference (2) - (1)
1.	45.5	45.7	+0.2
2.	43.3	42.9	-0.4
3.	41.2	40.9	-0.3
4.	44.0	44.2	+0.2
5.	38.7	38.2	-0.5
6.	40.9	41.4	+0.5

The most likely cause of the discrepancy is that none of the specimens of plasma were exactly isoplethechontic with a sodium chloride solution containing 0.871 g. NaCl per 100 ml.

We now return to the determination of R using the percentage cell volume which has been calculated

from protein analyses. (It makes very little difference to the calculated value of R whichever method for the percentage cell volume is used). From the percentage cell volume, we may calculate the relative volume of the cells at equilibrium in the blood-water system by equation xiii (p. 99). The tonicity of plasma is taken as unity; the tonicity of water is zero; and W is taken as 0.70.

In the second experiment, the calculated relative cell volume at equilibrium in the blood-water system is 1.54 times the initial volume.

The value of R is calculated thus:-

Plasma ..... 72.0 mg. protein/ml.

Plasma-water supernatant..... 40.7 mg. protein/ml.

72.0 mg. protein = 1.000 ml. plasma,

40.7 mg. protein = 0.565 ml. plasma.

But the total volume of plasma is 3 . 0.567 ml.

= 1.701 ml.

If 0.565 ml. plasma = 1.000 ml. supernatant,

1.701 ml. plasma = 3.010 ml. supernatant.

The volume of cells is therefore 5 - 3.010 ml.

= 1.990 ml.

The initial cell volume was 3 . 0.433 ml.

= 1.299 ml.

The relative equilibrium volume is therefore

$$\frac{1.990}{1.299} = 1.53$$

and $R = 0.53/0.54 = \underline{0.98}$.

The full results are given in Table 32.

Table 32. The Determination of R.

In all experiments, 2.00 ml. of saline (or water) is added to 3.00 ml. of blood.

Experiment No.	1	2	3	4	5	6
Plasma protein. mg. per ml.	58.4	72.0	59.0	64.85	60.1	66.4
Blood-saline supernatant protein. mg. per ml.	26.25	33.1	27.65	29.6	28.8	31.2
Blood-water supernatant protein. mg. per ml.	32.6	40.7	33.2	36.8	33.9	37.55
Percentage cells (calc.)	45.4	43.3	41.2	44.0	38.7	40.9
Tonicity (T).	0.450	0.459	0.469	0.457	0.479	0.470
Relative cell volume (calc.)	1.54	1.54	1.53	1.54	1.53	1.53
Relative cell volume (obs.)	1.52	1.53	1.51	1.55	1.50	1.52
R	0.96	0.98	0.96	1.02	0.94	0.98

Mean value of R = 0.973.

In all experiments, the ratio of blood to saline (or water) was 3:2. If a greater proportion of blood were used, the extent of swelling, and hence the accuracy of its determination, would be smaller. If a smaller proportion of blood were used, there

would be a danger of haemolysis which occurs when the cell increases to about 1.70 times its initial volume. The ratio of 3 volumes of blood to 2 volumes of water was a matter of choice and was selected as a matter of convenience. The value of R would approximate equally well to unity at any other ratio provided that the measurements were sufficiently accurate and provided that the extracellular fluid had an osmotic pressure high enough to prevent haemolysis.

The mean value of R is sufficiently close to unity to provide proof that the cell is a perfect osmometer under conditions of varying tonicity.

The Effect of Oxalate.

It has been mentioned above (p. 118) that Ponder (1943-44), using Evan's blue as the external "indicator", obtained good results with heparinised blood but not with oxalated blood. This he ascribed to the crenating effect of oxalate and proceeded to examine the phenomenon of crenation in search of the explanation. In 4 experiments with heparinised blood his mean value of R was 0.95, and in 12 experiments with oxalated blood his mean value of R was 0.73. Once again, the solution is not as difficult as it would appear and lies in a consideration of the amount of oxalate which is used.

The calcium of blood is confined entirely to the plasma which contains an average of about 10 mg. Ca per 100 ml. If the percentage cell volume is

40% cells, the whole blood will contain 6 mg. Ca per 100 ml. 6 mg. Ca is chemically equivalent to 20 mg. sodium oxalate. 5 ml. of blood therefore requires 1 mg. sodium oxalate to precipitate the whole of the calcium as oxalate. If this theoretical amount is used, the only osmotic effect will be the result of replacing one calcium ion with two sodium ions.

Ponder (1943-44), in a footnote, states

> "The quantities of sodium oxalate usually used
> "as an anticoagulant is 10 mg. per 5 ml. blood.
> "In these experiments, the quantity has been
> "doubled....."

If 20 mg. sodium oxalate is used to prevent the clotting of 5 ml. blood, 19 mg. of it will remain in the plasma - in solution and exerting an osmotic pressure. The effects of this will be obvious. The use of excessive amounts of oxalate is another source of trouble in the determination of relative cell volumes.

Part 7.

An interesting Application of Osmotic Effects.

The importance of purely osmotic phenomena is more than an exercise in physical chemistry. The following example illustrates very well the importance of osmotic pressure in the field of cell permeability.

When a substance enters a cell slowly, as, for example, glucose entering the erythrocyte, water also enters the cell in order to maintain osmotic equilibrium. The entry of the water, plus, of course, the glucose, produces cell swelling and eventual haemolysis. The course of the entry of the solute and water may be followed by recording either the rate of cell swelling or the rate of haemolysis. There is a very large literature on the subject and most of it is concerned with the rate of entry of glucose and other simple sugars into the erythrocyte. There are many theories about the precise mechanism of the transport of sugars across the cell wall and these point to the general conclusion that the mechanism is one of "active transport", i.e., an enzymic process with the phosphorylating enzymes as the suspected catalyst. Very briefly, the evidence for this is:-

1. the permeation of sugars does not obey the laws of simple diffusion,

2. there has been shown to be competition between simultaneously penetrating sugars,
3. the entry of sugars is completely stopped by the addition to the system of enzyme inhibitors such as phloridzin,
4. the transfer of sugar has a high temperature coefficient,
5. there is a high structural specificity of the sugars, and,
6. the analogy between the penetration of sugars into the erythrocyte and the transfer of sugars across natural membranes.

Although this appears to be a formidable list of criteria, all sound and experimentally correct so far as we know, there may be alternative explanations. In particular, the possibility of osmotic effects is generally ignored in spite of the fact that Klinghoffer (1935) clearly demonstrated that the higher the extracellular concentration of glucose, the slower the rate of entry of glucose into the erythrocyte. This at least raises the possibility of osmotic effects being concerned in the matter. The importance of osmotic pressure can be proved by the following experiment.

1.00 ml. of heparinised, oxygenated, human blood is added to 19.00 ml. of a solution containing 2.20 g. glucose per 100 ml. and the system is placed in a water bath at 20°C. with occasional gentle mixing. Samples are removed at suitable intervals for

determination of the degree of haemolysis.

Initially there is no haemolysis, but under these conditions, haemolysis begins after about 1 hour. Once the 10% haemolysis point is reached, the haemolysis curve is practically a straight line until about 60% of the cells have lysed. Thereafter, the rate of haemolysis gradually falls off and haemolysis is complete in about 3 hours (see Figure 15, graph ABC). The system is very sensitive to alterations in glucose concentration, pH, and particularly temperature, but the results are highly reproducible provided that these physical factors are kept strictly constant.

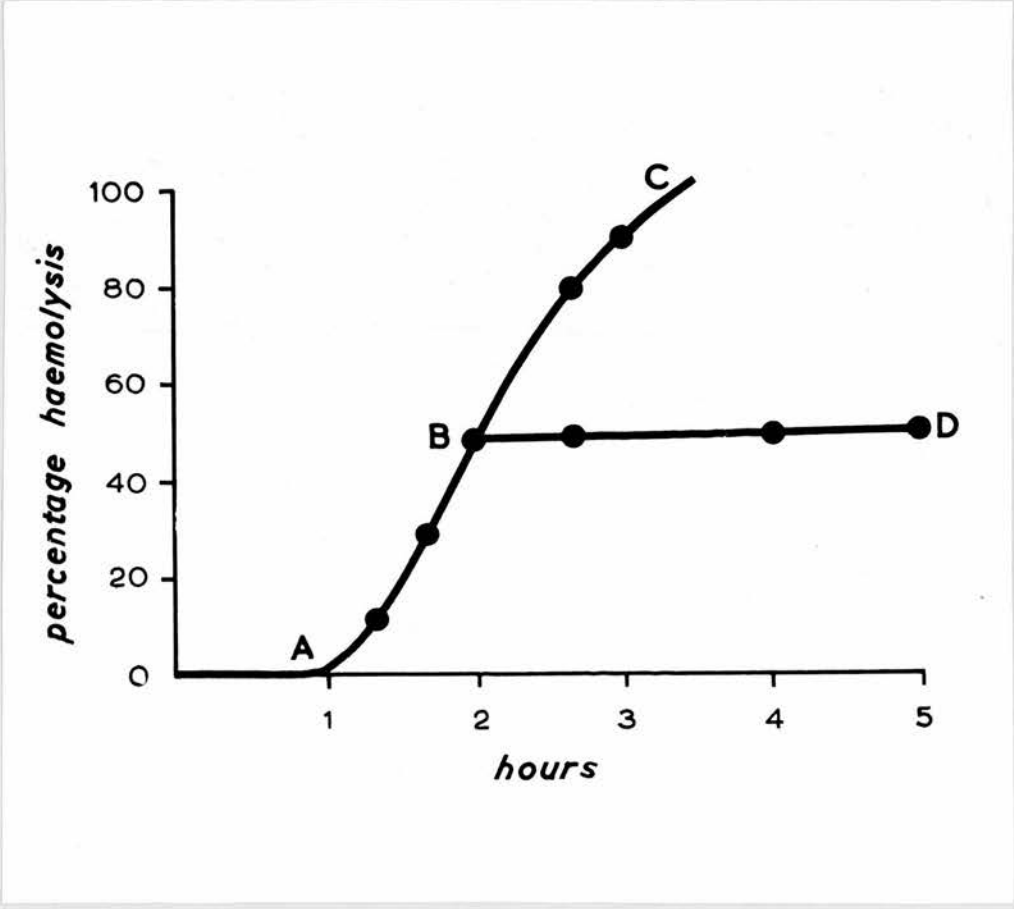

Figure 15. The rate of haemolysis of erythrocytes in dilute glucose solution.

If the haemolytic process is allowed to proceed until about half the cells have haemolysed (the exact percentage being accurately determined), and at that point enough solid fructose is added to the remainder of the system to double the osmotic pressure of the remaining extracellular fluid, haemolysis is abruptly, completely, and permanently stopped. (see Figure 15, graph ABD). Under the conditions of the experiment, fructose does not enter the human erythrocyte at any measurable rate.

Experiments of this type, in which the extracellular osmotic pressure is derived from more than one sugar, have been studied by LeFevre and Davies (1951) who concluded that the alteration in cell swelling (haemolysis) was evidence of substrate competition between the sugars for some enzyme system at the cell surface. If, however, in the above experiment, we replace the fructose by any other neutral compound which does not penetrate the cell, exactly the same result is obtained. The haemolytic process is completely stopped by the addition, at the point B, of mannitol, sucrose, sodium chloride, potassium bromide, or even glucose itself. The effect of the addition of more glucose brings us back in a roundabout way to the experiment of Klinghoffer, and the abrupt stoppage of haemolysis by sodium chloride effectively rules out any possibility of substrate competition. The stoppage of the haemolytic process (i.e., the entry of glucose into

the cell) by sodium chloride is of especial importance because many workers investigating the permeability of membranes to sugars use systems containing a high concentration of this salt.

The effect of the added fructose, sodium chloride, etc., is purely an osmotic effect: raising the extracellular osmotic pressure prevents the passage of water (plus glucose) into the remaining cells and so stops the haemolysis. On the other hand, if we add solid urea, urethane, thiourea, or acetamide at the point B, or indeed any other neutral substance which rapidly penetrates the erythrocyte membrane, then haemolysis proceeds along the graph ABC as if nothing had been added at all.

This simple experiment proves that in any study of the entry of slowly-permeating substances into the cell, the extracellular osmotic pressure is one of the critical and controlling factors, from whatever source this extracellular osmotic pressure may be derived.

Part 8.

The Osmotic Properties of Body Fluids.

The simplest direct method of determining the total osmotic pressure of any solution is to determine the freezing point, and the introduction of the Fiske Osmometer has eliminated most of the technical difficulties associated with the measurement of this quantity.

The Fiske Osmometer:

The essential heart of this instrument is a test tube into which is measured 2.0 ml. of the solution to be examined, and when fitted to the instrument, there dips into this solution (a) a highly stable electric thermometer in the form of a small thermistor, and (b) a vibrator rod of stainless steel about 1 mm. in diameter. The resistance of the thermistor is measured electrically in a Wheatstone Bridge circuit using the null balance method and the temperature of the thermistor is followed on a very sensitive galvanometer. A pump can be brought into operation which squirts intermittent jets of cold 50% aqueous ethylene glycol at about -6°C. over the outside of the tube. Briefly, the modus operandi is as follows:

(1) a switch sets the pump in operation and also sets the vibrator rod in motion so that the specimen is both cooled and gently stirred. The rate of cooling is followed by the movement of the galvanometer.

(2) as the temperature approaches the pre-selected degree of supercooling (which is arbitrarily taken as -20 divisions of the galvanometer scale) a second switch cuts out the cooling pump but allows the vibrator rod to continue mixing the sample. The rate of cooling is therefore slowed down so that the pre-selected degree of supercooling is gradually approached, and with a little practice the temperature at which the specimen is actually frozen can be reached to within $\pm$0.1 scale division, and held there for a few seconds.

(3) when this point is reached, a third switch sets the vibrator rod in violent motion for 2-3 seconds (after which it stops) and this induces freezing. The exact temperature of the mixture of ice and solution is then measured by the thermistor on a dial previously calibrated and measuring directly in milliosmols (mosm.). One milliosmol corresponds to a freezing point depression of 0.001859°C.

(4) the ice is then thawed out and the whole process repeated. A series of 5 readings can be made on any given solution in less than 15 minutes.

The great advantages of this instrument are:-

(1) the stirring (up to the point of freezing) is mechanical and the rate of stirring, and therefore the rate of cooling, are both regular.

(2) the degree of supercooling is accurately controlled and is highly reproducible - thereby

eliminating the greatest source of error in the conventional Beckmann thermometer method.

(3) the instrument (i.e., the milliosmol dial) is standardised by accurately prepared solutions of sodium chloride, and it can be re-standardised, or checked, in a few minutes at any time.

(4) the readings are highly reproducible and the error in any given estimation is less than ± 1 milliosmol. Since the total osmotic pressure of normal body fluids is about 300 milliosmols (per kg. water being understood) this represents an error not greater than ± 0.33%.

The standard solutions used for calibration contain sodium chloride and are prepared on the basis of the accepted data of the International Critical Tables or on the basis of the precision data of Scatchard and Prentiss (1933). Differences between molarity and molality have been ignored for the present purposes.

The Osmolarity of normal Serum.

Serum is the most readily available of all body fluids and acts as a standard of comparison both between different individuals and between different body fluids from the same individual. Most modern text-books give the freezing point of normal serum as -0.56°C. (301 milliosmols) but this osmolarity is about 4% too high.

Serum was collected from convalescent adult

patients who could reasonably be regarded as normal and from members of staff. The clotted blood could be kept at room temperature for at least 2 hours without affecting the serum osmolarity. Once the serum had been separated it could be stored at refrigerator temperature (4°C.) for several hours without change in osmolarity.

The mean value found in this series of 50 cases (25 male, 25 female) was 289 milliosmols (s.d. = 4). The osmolarity was the same for both sexes; the means and standard deviations were identical. This value is in good agreement with the results of other workers using different methods (see Table 33).

Table 33. The Osmolarity of Normal Human Serum.

		Osmolarity		
Number of Cases.	Method.	Mean.	S.D.	Reference.
30. M.	V.P.	286	6	(1)
20. F.	V.P.	287	6	(1)
21. M, F.	V.P.	289	6	(2)
28. M.	V.P.	288	4	(3)
75. M, F.	B.	291	8	(4)
50. M, F.	F.O.	289	4	(5)

References: (1) Culbert (1935), (2) Benham, Duke-Elder and Hodgson (1938), (3) Lifson (1944), (4) Olmstead and Roth (1957), (5) present work.
M = male, F = female. V.P.: vapour pressure method of Hill, B.: Beckmann thermometer method, F.O.: Fiske Osmometer.

The Osmotic Pressure of the Erythrocyte:

Evidence has been given in Part 6 to prove that the normal erythrocyte is a perfect osmometer. Further evidence relating to the erythrocyte in both normal and abnormal blood, can be provided by the Fiske Osmometer. One drawback of the osmometer is that it has great difficulty in handling specimens which are very viscous, such as a mass of packed, lysed, red cells. The reason for this is the difficulty of stirring such a specimen efficiently. All attempts to determine the total osmolarity of a mass of packed lysed erythrocytes, or the total osmolarity of a specimen of lysed polycythaemic blood, were failures. It is, however, possible to study the osmotic pressure of whole blood and its corresponding plasma and this method has the great advantage that cases can be selected to cover a wide range of variation in osmolarity. Although wide, the range is not entirely unlimited. Plasma osmolarity is seldom reduced below the normal limit of 282 mosm. and values below 260 mosm. are very rarely encountered (but one such case is given here). The reason for this is that if the serum total osmotic pressure falls significantly - which is virtually synonymous with a fall in the serum sodium and chloride concentrations - then urea and other end-products of metabolism are retained. At the other extreme, very large increases in serum osmolarity can, and do, occur. The highest value found in this

work was 457 mosm. in a very severe case of water depletion in which the serum sodium and chloride concentrations were 175 and 154 meq. per litre respectively and the serum urea was 308 mg. per 100 ml.

Venous blood was collected from a short series of patients, coagulation being prevented by the addition of a trace of the sodium salt of heparin. After 2.0 ml. of the whole blood had been pipetted into an osmolarity tube, the remainder was centrifuged. 2.0 ml. of the plasma was then transferred to a second osmolarity tube. To give an indication of the intracellular contribution to the whole blood osmolarity, the packed cell volume was also determined in each case (centrifuged for 30 minutes at 2,440 x g).

Attempts to haemolyse the whole blood specimens by addition of anionic detergents, saponin, etc., were singularly unsuccessful. The only methods of achieving complete haemolysis rapidly were to freeze the blood at -20°C. for 20-30 minutes in the deep-freeze, or to freeze the blood by immersion of the tube in a mixture of petroleum ether and carbon dioxide snow. With the former method, it was sometimes necessary to re-freeze the specimen before haemolysis was complete. Both of these methods have the additional advantage that no osmotically active substance (other than the heparin) is added to the blood. In all cases, the plasma specimen was also frozen in the same way, and for the same length of time, as the whole blood specimen. Results are

Table 34. Osmolarity of Whole Blood and its Plasma.

Case No.	Osmolarity (mosm/kg.water) Whole blood	Plasma	P.C.V. (%)	Clinical notes*
1	231	233	41.2	Adrenal failure; cause ? Na, 100; Cl, 83; urea, 22.
2	283	284	47.3	No clinical diagnosis.
3	285	287	32.3	Anaemia.
4	287	288	35.5	Anaemia.
5	295	297	42.5	Chronic bromism; Na, 141; Cl, 68; Br, 31.
6	321	323	16.7	Acute nephritis; severe anaemia; urea, 220.
7	333	333	43.3	Uraemia; patient being dialysed on artificial kidney; urea, 308.
8	355	356	42.5	Postoperative water depletion; Na, 168; Cl, 117; urea, 158.
9	362	363	33.2	Nephritis. Aldosteronism? Na, 157; Cl, 126; urea, 260.

* Urea given in mg. per 100 ml.; all others in meq. per litre.

given in Table 34.

The difference between the osmolarities of whole blood and the corresponding plasma is within the limits of experimental error, i.e. ± 1 mosm. for each of the two determinations, but the plasma osmolarity is the higher of the two except in one case where the blood was obtained from a patient in whom the artificial kidney was being used, and in this case it was unnecessary to add anticoagulant to the specimen since the patient was already heparinised. This suggests that the small difference between the osmolarities (the mean difference in the other 8 cases is only 1.5 mosm.) was at least partly due to the heparin. Heparin has a molecular weight of the order of 16,000 but it was used in the form of its sodium salt which ionises and which will, of course, remain in the plasma and contribute to its total osmotic pressure. Although only one type of cell, and a very specialised type, has been examined here, its behaviour has been studied over a wide range of osmolarity (130 mosm.) and the results show that there is no significant difference in osmolarity between whole blood and the corresponding plasma. In other words, the erythrocyte behaves as a perfect osmometer under conditions of abnormal blood chemistry.

True Body Fluids:

The existing classification of extracellular body fluids into "exudates", "transudates", "excretions", "secretions", "fluids", "juices",

"humours" and so on, merely confuses the issue since none of these terms has been accurately defined. For the present purposes, the term "true body fluid" or "body fluid" has been used to describe those extracellular fluids which occur normally, or which can accumulate abnormally, in the body cavities or tissues. Those studied were plasma (or serum), and ascitic, cerebrospinal, hydrocele, oedema, pericardial, pleural, spermatocele and synovial fluids, and a few miscellaneous cystic fluids. While most of these fluids occur normally in the body, only two are accessible in sufficient quantity for proper examination: the others, although unobtainable from the normal subject, also occur normally. The "fluid" in the normal pleural cavity consists of no more than a film separating the two pleural surfaces, and pleural fluid can be obtained in sufficient amount only from abnormal cases. For this reason, it is necessary in each case to compare the osmotic pressure of the fluid with that of the corresponding serum taken, as nearly as possible, at the same time.

Other Methods:

Sodium and potassium were estimated by the EEL flame photometer and chloride by the titrimetric method of Schales and Schales (1941). With a few highly pigmented fluids, the chloride method of Van Slyke (1923) had to be employed. Bicarbonate was determined manometrically using the Van Slyke

constant volume apparatus and converting the carbon dioxide liberated by lactic acid directly into meq. bicarbonate per litre. Total protein, and fractionation into albumin and total globulins, was carried out by the method of Gornall, Bardawill and David (1949). Some of the specimens contained appreciable concentrations of mucoprotein, and a few contained fibrinogen: these were not estimated separately. Urea was determined by the direct Nesslerisation method of Archer and Robb (1925).

In any specimen where a fibrin clot had formed, this was first removed by centrifugation. Some specimens contained a few erythrocytes which were also removed. Spermatocele fluid was centrifuged to remove spermatozoa, but it was found, in practice, that their presence made no difference to the observed osmolarity. In some of the body fluids, the rapid stirring of the vibrator rod required to induce freezing, resulted in the formation of a fibrin clot, but the molecular weight of fibrinogen is so very high that its conversion to insoluble fibrin cannot have anymeasurable effect on the total osmotic pressure. More serious trouble was experienced with very viscous fluids, especially with some of the specimens of synovial fluid. In spite of vigorous stirring, cooling tended to be irregular, and it was sometimes difficult to obtain repetitive accuracy with a few of the specimens. In some cases, it might be necessary to average a dozen or so

results on one single specimen.

Throughout this part of the work, all determinations of osmotic pressure are those of the total osmotic pressure; no measurements of the colloid osmotic pressure were made. The total osmotic pressure includes the colloid osmotic pressure which, in the case of normal serum, amounts to a little more than 1 mosm.

Clinical Material:

All specimens were obtained from hospital patients. Some were seriously ill with severe metabolic disturbances when the specimens were collected, and a few eventually died, but no specimen was collected from a moribund patient. The fluid and the corresponding serum were collected simultaneously whenever this was possible as, for example, in the drainage of oedema and ascitic fluids. In other cases, the time interval was reduced to a minimum - usually a matter of a few minutes. All the specimens were sterile. A few were necessarily obtained while the patient was under general anaesthesia. In a short series of cases, it was found that neither general anaesthesia nor major surgery to the point when the specimen was obtained, had any detectable effect on the serum osmolarity. The osmolarity of the serum on the morning of operation was within ± 1 mosm. of the osmolarity of the serum at that point during the operation when

the body fluid was reached. This fact suggests that body fluid osmolarity is not a quantity which changes rapidly under abnormal conditions.

The specimens were not collected under oil, and preservatives were not added. The serum was separated within 10 minutes and the bicarbonate concentration and osmolarity were determined without further delay.

Table 35. Osmolarity and Composition of Human Body Fluids.

A. Ascitic fluid.

Case. Diagnosis.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
7	Serum	289		130	-	92	-	3.2	2.4	5.6	108
Cardiac failure.	Fluid	289	0	135	-	92	-	1.9	1.2	3.1	114
11	Serum	290		138	-	96	-	3.5	3.1	6.6	44
Cirrhosis.	Fluid	290	0	138	-	103	-	1.1	0.5	1.6	44
29	Serum	274		135	4.5	94	-	3.3	1.6	4.9	30
Carc. breast.	Fluid	273	-1	135	4.2	103	-	2.1	0.8	2.9	26
36	Serum	294		137	3.8	103	-	4.2	2.5	6.7	44
Cirrhosis.	Fluid	293	-1	135	3.7	114	-	1.1	0.3	1.4	44
45	Serum	281		130	2.7	96	-	2.2	3.9	6.1	44
Cirrhosis.	Fluid	279	-2	135	2.6	97	-	0.4	0.9	1.3	39
57	Serum	264		126	5.3	95	21	2.8	3.1	5.9	26
Carc. breast.	Fluid	265	+1	126	4.0	99	22	0.6	0.7	1.3	22

A = Albumin G = Total globulins TP = total proteins. All in g. per 100 ml

U = Urea in mg. per 100 ml.

* denotes specimens obtained under anaesthesia.

Table 35 (contd.)

B. Cerebrospinal fluid.

Case. Diagnosis.		mosm.		Na	Cl	A	G	TP	U
4	Serum	292		138	97	4.0	2.2	6.2	66
Headaches.	Fluid	291	-1	143	119	-	-	0.07	60.
14	Serum	287		137	101	3.9	1.9	5.8	24
Convulsions.	Fluid	289	+2	140	119	-	-	0.02	24
22	Serum	289		140	104	5.0	1.3	6.3	-
? Syphilis.	Fluid	287	-2	144	131	-	-	0.02	-
24	Serum	292		138	101	4.5	2.0	6.5	32
Headaches.	Fluid	294	+2	138	121	-	-	0.04	33
33	Serum	288		130	89	4.7	2.1	6.8	72
? Brain tumour	Fluid	286	-2	135	115	-	-	0.03	63
42	Serum	285		135	98	4.5	2.0	6.5	21
Brain stem thrombosis.	Fluid	284	-1	135	116	-	-	0.07	24.

Table 35 (contd.)

C. Hydrocele fluid.

Case.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
26	Serum	290		137	4.1	98	-	4.1	2.7	6.8	38
	Fluid	289	-1	135	4.0	100	-	3.9	1.5	5.4	36
32	Serum	288		136	4.0	100	-	4.4	1.7	6.1	37
	Fluid	288	0	138	3.9	105	-	1.7	0.4	2.1	41
38	Serum	298		135	3.7	108	-	4.5	2.2	6.7	76
	Fluid	296	-2	135	3.8	109	-	3.2	0.9	4.1	78
39	Serum	289		135	4.1	102	-	4.5	2.1	6.6	35
	Fluid	290	+1	135	3.9	108	-	3.2	0.8	4.0	33
41	Serum	293		137	3.3	106	-	5.1	2.0	7.1	44
	Fluid	292	-1	137	3.3	110	-	4.6	1.1	5.7	37
60	*Serum	291		135	4.3	108	25	3.6	2.1	5.7	41
	*Fluid	292	+1	138	3.9	109	25	3.5	1.0	4.5	37

Table 35 (contd.)

D. Oedema fluid.

(oedema fluid removed from both legs simultaneously by Southey's tubes).

Case. Diagnosis.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
1	Serum	283		122	-	85	-	3.9	2.1	6.0	166
Cardiac failure.	R. leg	284	+1	122	-	85	-	-	-	0.4	162
	L. leg	284	+1	122	-	85	-	-	-	0.4	158
2	Serum	295		135	-	99	-	2.6	4.2	6.8	148
Cirrhosis.	R. leg	294	-1	130	-	101	-	-	-	0.6	165
	L. leg	293	-2	130	-	104	-	-	-	0.6	152
12	Serum	310		130	-	96	-	2.7	1.2	3.9	292
Acute nephritis.	R. leg	311	+1	130	-	101	-	-	-	0.4	304
	L. leg	308	-2	130	-	101	-	-	-	0.3	292
58	Serum	283		130	4.4	92	27	3.7	2.0	5.7	72
Cardiac failure.	R. leg	282	-1	133	4.2	97	31	-	-	0.3	72
	L. leg	282	-1	133	4.2	97	31	-	-	0.2	72
62	Serum	314		135	-	112	24	2.7	3.7	6.4	125
Nephritis and	R. leg	312	-2	138	-	120	21	-	-	0.3	142
diabetes.	L. leg	312	-2	138	-	120	22	-	-	0.3	142
71	Serum	287		130	4.5	101	26	3.4	1.4	4.8	34
Diffuse	R. leg	287	0	130	3.9	109	30	-	-	0.4	30
Carcinomatosis.	L. leg	287	0	136	4.1	110	29	-	-	0.5	34

Table 35 (contd.)

E. Pericardial fluid.

Case.	Diagnosis.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
	51	*Serum	288		135	4.2	106	23	-	-	6.8	30
Mitral	stenosis.	*Fluid	288	0	137	4.0	109	27	-	-	3.6	30
	68	*Serum	286		130	3.4	100	25	3.7	1.6	5.3	25
Mitral	stenosis.	*Fluid	286	0	130	3.4	105	30	1.9	0.5	2.4	25
	69	*Serum	287		136	4.0	94	-	4.2	2.0	6.2	36
Mitral	stenosis.	*Fluid	288	+1	136	4.0	101	-	2.3	0.7	3.0	34
	70	*Serum	290		136	4.5	100	-	4.2	1.9	6.1	32
Mitral	stenosis.	*Fluid	290	0	136	4.1	107	-	1.5	0.5	2.0	32
	72	*Serum	279		128	3.4	105	22	3.8	1.7	5.5	28
Mitral	stenosis.	*Fluid	280	+1	128	3.5	106	26	2.6	0.7	3.3	26
	79	*Serum	281		136	4.4	104	18	4.3	1.3	5.6	21
Mitral	stenosis.	*Fluid	282	+2	136	4.3	103	25	1.8	0.5	2.3	19

Table 35 (contd.)

F. Pleural fluid.

Case. Diagnosis.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
5	Serum	288		135	-	99	-	-	-	6.5	37
Lung infarct.	Fluid	287	-1	135	-	104	-	-	-	4.4	37
16	Serum	286		138	4.5	103	-	3.7	2.6	6.3	34
Reticulosis.	Fluid	288	+2	138	3.9	107	-	2.6	1.3	3.9	32
43	Serum	282		135	4.0	98	-	4.0	2.1	6.1	30
Carc. breast.	Fluid	282	0	135	3.6	102	-	3.2	1.3	4.5	30
49	Serum	277		126	3.3	90	30	3.1	2.3	5.4	48
? Bronchitis +	Fluid	277	0	135	3.2	97	28	1.9	1.3	3.2	48
55	Serum	285		136	4.3	98	-	4.5	3.1	7.6	35
No diagnosis.	Fluid	282	-3	135	3.4	98	-	2.7	0.9	3.6	37
66	Serum	284		136	4.0	94	25	3.9	1.7	5.6	32
No diagnosis.	Fluid	282	-2	130	3.5	103	21	2.3	1.0	3.3	32

Table 35 (contd.)

G. Spermatocele fluid.

Case.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
31	Serum	292		138	4.2	106	-	4.9	2.1	7.0	43
	Fluid	292	0	138	5.3	135	-	-	-	0.13	41
53	Serum	289		135	4.2	104	29	4.4	1.8	6.2	41
	Fluid	287	-2	135	5.5	139	6	-	-	0.20	43
54	*Serum	291		136	4.0	102	27	4.1	2.1	6.2	37
	*Fluid	289	-2	138	4.7	133	3	-	-	0.42	37
56	Serum	287		135	4.1	96	27	4.4	2.3	6.7	46
	Fluid	287	0	135	4.5	133	11	-	-	0.14	48
59	Serum	293		135	3.9	107	24	4.2	2.9	7.1	37
	Fluid	294	+1	138	4.8	143	5	-	-	0.25	48
65	*Serum	293		141	3.6	98	25	4.5	1.5	6.0	29
	*Fluid	295	+2	141	5.9	131	3	-	-	0.48	34

Table 35 (contd.)

H. Synovial (knee-joint) fluid.

Case. Diagnosis.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
30	Serum	293		138	5.3	103	27	4.5	1.8	6.3	48
Osteoarthritis.	Fluid	291	-2	122	3.8	104	21	2.5	0.3	2.8	44
48	Serum	293		138	4.2	104	28	4.4	2.1	6.5	28
Post-trauma.	Fluid	290	-3	118	3.1	106	21	2.0	0.6	2.6	26
61	Serum	292		138	3.9	103	28	3.9	2.2	6.1	28
Post-trauma.	Fluid	293	+1	115	3.3	108	20	2.3	0.7	3.0	28
64	Serum	290		136	4.7	94	33	3.4	1.8	5.2	33
Osteoarthritis.	Fluid	288	-2	130	3.8	106	21	2.1	1.8	3.9	33
75	Serum	290		130	4.3	97	26	4.4	1.7	6.1	21
? Cause.	Fluid	287	-3	126	3.7	95	21	3.1	0.8	3.9	19
78	Serum	284		130	3.7	93	26	4.1	2.2	6.3	30
Rheumatoid arthritis.	Fluid	285	+1	115	3.7	90	24	3.4	1.0	4.4	25

Table 35 (contd.)

I. Spermatocele fluid and Hydrocele fluid.

(from patients having a hydrocele and a spermatocele simultaneously).

Case.		mosm.		Na	K	Cl	A	G	TP	U
	Serum	291		135	3.8	100	4.2	2.3	6.5	52
40	*Hydrocele	290	-1	136	4.5	108	3.1	1.1	4.2	55
	Spermatocele	292	+1	136	5.3	138	-	-	0.80	45
	*Serum	290		138	4.2	106	4.2	2.0	6.2	32
47	*Hydrocele	289	-1	138	4.1	110	3.3	0.9	4.2	37
	*Spermatocele	291	+1	143	3.9	116	1.5	0.3	1.8	38

Table 35 (cont.)

J. Miscellaneous Fluids.

Case. Diagnosis.		mosm.		Na	K	Cl	HCO_3	A	G	TP	U
50	*Serum	293		138	4.1	104	24	4.8	1.5	6.3	24
Thyroid cyst.	*Fluid	289	-4	135	3.7	106	19	3.0	5.1	8.1	24
52	*Serum	283		138	4.6	106	27	4.5	1.6	6.1	24
Pancreatic cyst.	*Fluid	285	+2	143	3.7	103	38	-	-	0.12	18
73	*Serum	286		136	4.1	98	26	3.5	1.7	5.2	25
Breast cyst.	*Fluid	287	+1	136	5.3	104	22	2.4	1.1	3.5	32
74	*Serum	288		135	4.2	104	27	3.8	1.4	5.2	22
White bile.	*Fluid	287	-1	136	3.8	126	3	2.9	0.9	3.8	26

The greatest difference observed between the osmolarity of a body fluid and that of the corresponding serum was + 4 mosm. in the fluid obtained from the thyroid cyst (case 50). This fluid was exceedingly viscous due to mucoprotein and altered blood which made efficient stirring difficult and the results erratic. But in spite of the cyst having been the site of a recent haemorrhage, the cyst fluid must be regarded as a true body fluid since it is a loculated fluid in equilibrium with the surrounding tissues and fluids. In three other cases, the difference in osmolarity was just outwith the probable error, - 3 mosm. in cases 48, 55, and 75. In all other comparisons, the difference was ± 2 mosm. or less.

There were 20 positive differences, 30 negative differences, and 12 zero differences in the series of 62 comparisons. The distribution of positive and negative differences is not believed to be significant. The mean arithmetic difference in the 62 comparisons was 1.2 mosm.

Apart from the 4 cases mentioned above, where the differences between the osmolarity of the body fluid and that of the corresponding serum were greater than would be expected, the only other divergent results were found in some cases of oedema fluid. If the osmolarities of the serum and of the oedema fluid are measured soon after the Southey's tubes are inserted, any difference is within the

limit of experimental error (see Table 35, D). Once the tubes are inserted, it is customary to leave them draining for several days. If specimens are collected 36-48 hours after insertion of the tubes, the oedema fluid may not have the same osmolarity as that of the serum, and differences of the order of 4-6 mosm. between fluid and serum collected simultaneously have been found. The cause of this has not yet been discovered.

Since a fairly wide range of osmolarity has been covered in these cases (minimum, 264; maximum, 314 mosm.) the conclusion has been reached that any true body fluid, normal or abnormal, is isosmotic with the corresponding serum, and therefore, by inference, all true body fluids are isosmotic with each other.

In 9 of the 54 patients, the serum osmolarity lay outside the normal range of 282-296 mosm. But abnormal chemical composition is not necessarily accompanied by an abnormal osmolarity; a decrease in serum sodium and chloride concentrations may be, and frequently is, compensated by an increased concentration of nitrogenous substances (e.g., cases 1 and 7), but this is not necessarily a matter of cause and effect. On the other hand, whereas the chemical analyses may indicate an abnormal osmolarity, the observed osmolarity may be normal or near normal (e.g., cases 2, 7, 58) - presumably as a result of compensation by undetermined constituents of the serum or body fluid. For the same reason, there is

no correlation between the sum of the cations (Na and K) and the sum of the anions (Cl, HCO_3 and protein) which have been estimated in these experiments, and very little is known about the concentrations (relative to serum) of the other ions present in these body fluids.

Chemical Differences:

The most significant differences in sodium concentration were those between synovial fluid and the corresponding serum where, in the 6 cases, the synovial fluid sodium concentration was an average of 14 meq. per litre lower than that of the serum. This reduction is partly, at least, balanced by a reduction in the bicarbonate concentration. With the other fluids, the difference in sodium concentration, compared with the corresponding serum, was 5 meq. per litre or less in all but three instances (cases 49, 66, 71).

Differences in potassium concentration were small; the difference between the concentration in the fluid and in the corresponding serum was less than 1.0 meq. per litre in 37 of the 45 comparisons in which this estimation was carried out. The finding of a higher concentration of potassium in seven of the eight spermatocele fluids compared with the corresponding serum, might be mentioned in passing, since, in the other comparisons, the serum potassium concentration was usually the higher.

In only 4 of the 62 comparisons was the chloride concentration less than that of the corresponding serum (cases 53, 75, 78, 79). The increased chloride concentration in cerebrospinal fluid has long been known, and in the 6 cases studied here, the mean difference in chloride concentration between serum and cerebrospinal fluid was 22 meq. per litre. A surprising discovery was that an even greater difference is to be found between the chloride concentration of spermatocele fluid and that of the corresponding serum: in the 8 comparisons recorded here, the mean difference was 31 meq. per litre. It is generally believed that the high chloride concentration in cerebrospinal fluid is a direct consequence of the low protein concentration, but the concentration of protein in spermatocele fluid is about 10 times that of cerebrospinal fluid, yet the difference in chloride concentration between spermatocele fluid and serum is much greater than the same difference between cerebrospinal fluid and serum (see Table 36). Again, although the protein concentration of oedema fluid is also about 10 times that of cerebrospinal fluid, the difference in chloride concentration between oedema fluid and that of the corresponding serum is very small (see Table 36). Only one case of mucocele of the gall bladder (a comparatively uncommon condition) was available in this series, but the biochemical resemblance (e.g. electrolyte concentrations) of this fluid to

spermatocele fluid is quite striking. Yet this mucocele fluid had a protein concentration about 10 times that of spermatocele fluid and about 100 times that of cerebrospinal fluid. If the Donnan membrane phenomenon is the cause of the relatively high chloride concentration of cerebrospinal fluid, then it is clear that it does not operate in the mechanism of the accumulation of spermatocele fluid, oedema fluid, or white bile.

Table 36. Summary of the Protein and Electrolyte Concentrations of Body Fluids compared with the corresponding Serum.*

Fluid and number of specimens.	Mean total protein.	Mean difference from corresponding serum.			
		Na.	Cl.	HCO_3.	
	g./100ml.	meq. per litre.			
Cerebrospinal (6)	0.04	+3	+22	-	(1)
Pancreatic cyst (1)	0.12	+5	- 3	+11	
Oedema (12)	0.39	0	+ 5	-	(2)
Spermatocele (8)	0.53	+1	+31	-21	(3)
White Bile (1)	3.8	+1	+22	-24	

* Compiled from the data in Table 35.

(1) Difference in bicarbonate known to be small.

(2) Difference in bicarbonate variable but small (-1 to +4).

(3) Mean of 5 differences in bicarbonate conc.

Unfortunately, few bicarbonate analyses were carried out until an explanation was being sought for the high chloride concentration in spermatocele fluid. Although only five analyses are recorded, the very low bicarbonate concentration in spermatocele fluid is most conspicuous, and since the sodium concentration does not differ significantly from that of the corresponding serum, the most likely explanation is that the membrane involved is capable of replacing bicarbonate with chloride by some mechanism unknown. Spermatocele fluid is always more acid than the corresponding serum; the pH of the fluids shown in Table 35 ranged from 6.2 to 7.1. The one available specimen of white bile was also acid - pH, 6.47. The small volumes of cerebrospinal fluid available precluded analysis for bicarbonate in this series, but it is recorded in most standard textbooks that the bicarbonate concentration of cerebrospinal fluid does not differ significantly from that of the corresponding serum and this fact has been verified in other experiments not connected with this work. In synovial fluid, the relatively low bicarbonate concentration is presumably related in some way to the low sodium concentration.

While cerebrospinal, oedema, spermatocele and pancreatic cyst fluids approximate to "protein-free" ultrafiltrates, the other fluids do not, and in particular, the concentrations of protein in hydrocele and pleural fluids are considerable (the

mean values in this series were 4.3 and 3.8 g. per 100 ml. respectively). In two cases (nos. 45 and 57) where the albumin:globulin ratio was reversed in the serum, it was also reversed in the body fluid - ascitic fluid in both cases. In only one case, that of the thyroid cyst fluid, was the protein concentration of a body fluid greater than that of the corresponding serum, and in this particular case, the cause was an old haemorrhage.

Of special interest were those two cases where the patients had a hydrocele and a spermatocele at the same time and specimens of both fluids were obtained. The situation is that of one abnormal fluid collection (the spermatocele) surrounded by a second abnormal fluid collection (the hydrocele) and both are in osmotic equilibrium with a third body fluid (the plasma). Although quite different in chemical composition, the osmolarity is the same in all three fluids.

These analytical observations are not intended either to be fully representative of the body fluids studied, or to be equated with the observed osmolarities. Those constituents which have been determined represent only a fractional contribution to the total osmotic pressure, and while, in some cases, the sum of the sodium plus the potassium is greater than the chloride plus the bicarbonate, in others, it is the reverse.

The Osmolarity of other biological Fluids:

A few preliminary investigations have been made on other biological fluids. The osmolarity of urine can vary from 50 to 1,200 mosm. without difficulty depending on the state of the body's water metabolism. Gastric juice has a variable total osmotic pressure, usually being hypotonic to serum. Saliva and sweat are always considerably hypotonic. Hepatic bile has a total osmotic pressure very near that of the corresponding serum, but lower. These fluids are not in osmotic equilibrium with the extracellular fluid of the body proper and are probably best described as "excretions" to distinguish them from true body fluids.

Discussion.

The material of this thesis falls into two distinct parts which are complementary since we cannot consider the osmotic, or haemolytic, properties of a cell without at the same time considering the osmotic properties of the extracellular fluid. In dealing with osmotic properties, it has been found safer to start with the established laws of physical chemistry and apply them to the behaviour of the cell since we know far more about physical chemistry than about cells. This approach has yielded dividends, particularly in the elucidation of the effects of change of temperature on osmotic swelling and haemolysis.

The existence of haemoglobin attached to the red cell membrane and not liberated into the supernatant fluid of the haemolysing system, has been postulated on many occasions. Such an occurrence would severely affect the accuracy of the method of determining the degree of haemolysis. Any haemoglobin which might be firmly attached to the membrane is so small in amount that it cannot possibly affect the method of measurement (pp. 4,5), and it is evident from the data in Tables 1 and 2 (pp. 7,8) that any error from this source is insufficient to influence the conclusion that haemolysis is an all-or-none process.

The difficulties of preparing haemoglobin-free

ghost cells in quantity seem to stem from the standard method of preparing ghosts for experimental work. The usual practice is to add blood to a small volume of water, allow haemolysis to proceed to completion, and then add solid sodium chloride to raise the osmotic pressure to normal, shrink the ghosts, and retard stromatolysis. It follows that, at the point when the solid sodium chloride is added, the haemoglobin will be equally distributed between the extracellular and the intracellular fluids (since the membrane is no longer functional), and when the ghosts are thus reconstituted, a certain amount of haemoglobin will be trapped inside them. This process constitutes the so-called "reversal of haemolysis" - an idea which was very popular at one time but which has now been abandoned. That this is the true explanation, was shown by Parpart (1942) whose method of preparing haemoglobin-free ghost cells consisted of adding the blood to a very large volume of water, and thus reducing the concentration of trapped haemoglobin.

The slow increase in the degree of haemolysis which occurs in a partially lysed system (Table 3, p. 11) is of no more than passing interest. It merely indicates that here is a variable to be controlled. In any experiment, the time selected for the duration of haemolysis is a matter of convenience. In the data in Table 24 (p. 72) and in Table 25A (p. 78) the results in each are all

strictly comparable; but the two tables are not comparable with each other since in the former, the duration of haemolysis was 1 hour, and in the latter, only 5 minutes.

The decrease in fragility which occurs when blood is allowed to stand with occasional mixing (Table 4, p. 13) is a very different matter. With venous blood, the change is considerable; with oxygenated blood, it does not occur. The remarkable and quite unexpected finding that "de-oxygenation" had the same effect on fragility as oxygenation, clearly pointed to the carbon dioxide of the blood being the critical factor, and eliminated at least one possibility, namely, that the increase in fragility of venous blood might have been associated with the formation of pyruvic and lactic acids. The increase in pH which occurs after both oxygenation and de-oxygenation, and the fact that both venous and oxygenated blood have the same fragility when the pH is controlled (Table 7, p. 21), prove that pH is the governing factor, and the only possible explanation is loss of carbon dioxide.

The experiments in which carbonic anhydrase was inhibited by the addition of sulphonamide were only a qualified success (Table 8, p. 25) due to technical difficulties. Sulphanilamide, which is a very powerful inhibitor of carbonic anhydrase, is only slightly soluble, and with difficulty at that. The

sulphanilamide must be added to, and dissolved in, the whole blood (not the hypotonic saline solution) and the dissolving of the sulphanilamide by mixing and shaking generates those very conditions which the inhibitor is supposed to prevent - loss of carbon dioxide. Nevertheless, the results are sufficiently conclusive and there can be no doubt that this phenomenon is related to loss of carbon dioxide catalysed by the activity of carbonic anhydrase.

These experiments provide the explanation for the observation by Whitby and Hynes (1935), and several others, that venous and arterial bloods have different fragilities. They explain the observation that venous stasis increases red cell fragility (Cormick, 1942), which it does by increasing the carbon dioxide content, and also the findings that the red cell fragility is increased in pneumonia (Needles, 1936), in congestive cardiac failure (Waller, 1939), in anoxia and carbon dioxide inhalation (Booth, 1941), and various other observations all associated with lowering of the blood pH.

The loss of carbon dioxide during the mixing of a blood sample raises many points of practical importance both in biochemistry and in haematology. In the determination of the fragility of red cells as a clinical procedure, the "venosity" of random samples of blood varies considerably, as every clinician knows. So also will the fragility vary.

If finer departures from the normal are to be examined, preliminary oxygenation is essential; the clinical value of such a refinement is quite unknown.

Loss of carbon dioxide also affects the packed cell volume of blood (Table 29, p. 106), and this factor should be controlled if the mean corpuscular haemoglobin concentration is to be determined accurately. Thus, compare the values given for blood D in Table 29:

	Hb.	P.C.V.	M.C.H.C.
Venous	14.6	45.8	31.9
Oxygenated.	14.7	43.3	34.0

At slightly lower levels, such differences would be clinically significant and might even determine the form of treatment. In practice however, the amount of mixing required before sampling for analysis is considerable, and all specimens are thereby reduced to roughly the same degree of oxygenation. Because of this, the mean corpuscular haemoglobin concentration in any individual is fairly constant. The following values were obtained in a case of pernicious anaemia under satisfactory therapeutic control over a period of two years:

Case. J.R. Male. aet. 52. Venous blood.

Date.	M.C.H.C.	Variation from Mean.
18. 5.53.	33.5	-0.3
13. 7.53.	34.1	+0.3
7. 9.53.	33.2	-0.6
5.10.53.	32.8	-1.0
23.11.53.	33.5	-0.3
18. 1.54.	34.6	+0.8
12. 4.54.	34.1	+0.3
5. 7.54.	33.3	-0.5
4.10.54.	33.8	nil
17. 1.55.	34.1	+0.3
4. 5.55.	34.6	+0.8
Mean.....	33.8	

The variation in fragility with pH at constant osmotic pressure (Figure 8, p. 57) is of obvious practical importance, and the rapid change in fragility in the range pH 7.2 to 7.5 is the most important point. A full explanation of the observations is not possible from the available data. The occurrence of a maximal fragility at pH 6.75 - 6.85 is of dubious physiological importance since this pH range is so far removed from the normal. The fact that the isoelectric point of haemoglobin coincides with this maximum, is probably mere chance. The breakdown of the erythrocyte membrane is far more likely to be associated with a physical or chemical change in the membrane itself, than with a change in the physical state of the intracellular haemoglobin. The effect on the erythrocyte membrane of lowering the pH of the extracellular fluid to about 6.8, is unknown. The isoelectric point of stromatin is said to be about 1.7 (Ponder, 1948, p. 135) which is rather a surprising figure, even

when the high concentration of phospholipoid at the cell surface is taken into account. Further argument would serve no useful purpose since the conditions of the experiments are so unphysiological. The really important point here is the change in fragility within the pH range of 7.2 to 7.5, all other factors having been controlled.

The effect of altering the electrolyte in the haemolysing system has provided some interesting results. Unfortunately, comparison with the results of other workers is rarely possible due to the differing experimental conditions.

Haemolysis in solutions of sodium fluoride is outstandingly different from haemolysis in solutions of any of the other salts. There are certain possible explanations of this, but in the writer's view, the fact that sodium fluoride is a powerful enzyme poison, is not one of them although this idea has been put forward by others. There is no evidence of any kind that enzymic activity plays any part in osmotic haemolysis. The osmotic data for sodium fluoride are not very good and this is possibly connected with the difficulties of purifying the salt which does not crystallise well. Whatever the cause, the recorded data for freezing point depression and electrical conductivity are in poor agreement. Hydrofluoric acid is markedly weaker than any of the other halogen acids and the sodium

salt must therefore be subject to salt hydrolysis, although its solutions are not markedly alkaline. If we omit sodium fluoride from the discussion, the osmotic pressure of solutions of the other salts at which 50% haemolysis occurs may be re-tabulated thus (cp. Table 25, p. 73):-

	Osmotic pressure. (atmos.).	Difference from the mean.
Potassium chloride	3.01	+0.19
Rubidium chloride	2.92	+0.10
Potassium bromide	2.88	+0.06
Sodium chloride	2.81	-0.01
Potassium iodide	2.80	-0.02
Sodium bromide	2.78	-0.04
Caesium chloride	2.77	-0.05
Sodium iodide	2.72	-0.10
Lithium chloride	2.67	-0.15
Mean	2.82	
S.D.	0.10	
Mean $\pm$ 2 S.D.	2.62 to 3.02	

For what statistical treatment is worth in so small a series, there is no significant difference between the osmotic effects of these 9 salts. Yet, when the matter is re-examined, eliminating one possible variable, it appears that there <u>is</u> a real difference between the osmotic effects of these salts (cp. Table 25A, p. 78). The difference, however, is small. For example, in the last block of data in Table 25A (osmotic pressure = 2.60 atmospheres) a change of 0.04 atmospheres pressure would alter the degree of haemolysis by 10%. It would, perhaps, be carrying the application of mathematics too far to point out that the average percentage haemolysis for the 12 blood specimens in each of the 5 salt solutions in Table 25A comes out in precisely the

same ratio as the ratio of the osmotic pressure of the same five salt solutions required to produce 50% haemolysis as recorded in Table 25.

There are two conclusions which may be drawn from these results.

First, the erythrocyte is not entirely indifferent to the nature of the salt providing the extracellular osmotic pressure. To demonstrate this, some selection of data is necessary, e.g., potassium chloride compared with sodium iodide, or rubidium chloride compared with lithium chloride. Why there should be these differences is not known.

Secondly, when the whole series of nine salts is examined, it must be admitted that the difference between the osmotic effects is very small, and in the case of five of them (KBr, NaCl, KI, NaBr and CsCl) the difference is virtually nil. These salts contain three different cations and three different anions. If the experiments had been confined to these five salts only, and they would have provided a reasonably fair selection, one could have safely concluded that the erythrocyte is completely indifferent to the nature of the extracellular electrolyte. This would have been a very important conclusion since a perfect osmometer is necessarily indifferent to the origin of the osmotic pressure, provided that no chemically reactive substances are involved. As things stand, this conclusion is very nearly, but not

quite, justified. It is unfortunate that the differences between the effects of these 9 salts are too small to be taken seriously, and too large to be ignored.

The only other paper to be found in the literature dealing with haemolysis in solutions of alkali halides is one by Ponder (1948-49) which appeared at about the same time as the writer's own paper on the subject (Hendry, 1948). Ponder used the five alkali chlorides and his results are expressed in terms of the tonicity (plasma = 1.00) required to produce 50% haemolysis. His results compared with author's results (which have been converted into tonicities for comparison) are:-

	Ponder (1948-49)	Hendry (1949)
Lithium chloride	0.53	0.38
Sodium chloride	0.45	0.40
Potassium chloride	0.49	0.43
Rubidium chloride	0.49	0.42
Caesium chloride	0.47	0.40

The difference in results is not surprising in view of the different conditions at which the 50% haemolysis point was determined. This is a very good example of the great difficulties of comparing the work of different observers (see pp. 62 et seq.) Ponder, in his experiments, used washed red cells and the final dilution was 1 in 250; the temperature was 22°C.; the anticoagulant used was heparin; and the time for which haemolysis was allowed to proceed was 5 hours.

The general conclusion reached in this section

of the work is that the erythrocyte is almost (but not quite) indifferent to the nature of the extracellular electrolyte.

The various theories which have been put forward to account for the effect of change of temperature on the fragility of erythrocytes have already been summarised (pp. 60-61, 82-83). One of these theories, that of Jarisch is disproved by the observation (Table 22, p. 60) that lowering the temperature of a system containing erythrocytes suspended in a phosphate buffer still produces a considerable increase in the degree of haemolysis. Ponder's theory is also disproved by the known fact that haemolysis is almost instantaneous whereas the leakage of intracellular material is relatively a very slow process.

When erythrocytes are suspended in hypotonic saline, potassium escapes from the cell. This was shown conclusively by Davson (1940) and has been confirmed by others. Davson showed that for six different species, the rate of escape of potassium was more rapid at 40°C. than at 5°C. With human erythrocytes (initial potassium content = 100) he showed that the potassium remaining intracellularly after one hour at 40°C. was 94.5, and at 5°C. was 97.3. The rate of haemolysis of erythrocytes in water or very dilute saline is a difficult quantity to measure accurately: Fricke (1935) found that lysis was complete in 0.16 second, and other estimates

are of the same order. The comparatively slow escape of intracellular potassium cannot therefore be an explanation of any of the factors involved in cell swelling or haemolysis.

The persistence of the temperature effect even when the pH of the system is controlled (p. 60) argues against changes in the base-binding capacity of haemoglobin being a factor. All available evidence points to the intracellular pH being the same as the extracellular pH - hydrogen and hydroxyl ions passing through the cell membrane more rapidly than any other ion - so that when cells are suspended in a buffer medium of constant pH any change in the degree of ionisation of the haemoglobin must be very small and quite insufficient to account for the observed change in osmotic fragility.

It is surprising that more attention has not been paid to the effect of change of temperature on the osmotic pressure of the haemolysing medium. No reference to this can be found in the literature on haemolysis or cell swelling, in spite of the fact that haemolysis in hypotonic salt solution has long been referred to as "osmotic haemolysis". The effect of change of temperature on the osmotic pressure of a solution was first described by van't Hoff in 1886.

It has been shown by calculation (pp. 83-85) that the change in osmotic pressure of the system due to a given change of temperature is in the same direction, and is of approximately the same magnitude,

as would account for the observed change in erythrocyte fragility. It is then an easy matter to prepare a series of solutions which will have the same osmotic pressures at different pre-selected temperatures, and to show that, at constant osmotic pressure, the fragility of the erythrocytes is independent of temperature (Table 27, p. 95). The results are conclusive, and while the verification of theory by experiment is a satisfying experience, the real importance of these findings lies in the fact that the theory demanded that the erythrocyte should act as a perfect osmometer. It may then be further concluded that the erythrocyte does, in fact, behave as a perfect osmometer under the conditions of the experiments - namely, change of temperature.

The experiments in Part 6 need little elaboration. The data in Table 30 (p. 115) prove that any value of R between 1.69 and 0.82 can be obtained at will by altering the centrifugal force, and the key to this is contained in the last sentence of the quotation from Ponder given on p. 113. It is surprising that so much work has been put into a method which has such a palpable defect, and yet it is easy to understand why such a standard method as the haematocrit method for determining packed cell volumes came to be applied without question to systems containing artificially swollen cells. The ingenuity that has been displayed in explaining the significance of the factor "R" is also remarkable. At one time or another it has been

explained in terms of loss of intracellular potassium prior to haemolysis, to the occurrence of bound water in the erythrocyte, in spite of the work of A. V. Hill (1930), to change in the red cell interior due to gelation, to the abnormal relation between the concentration and the osmotic coefficient of haemoglobin, to the elastic properties of the erythrocyte membrane, and so on. But "R" was never anything more than a "correction factor". The conclusion that the red cell is a perfect osmometer is based on the following:

1. There is no a priori reason to believe that the red cell membrane would be other than a true semi-permeable membrane obeying the laws of physical chemistry.
2. The low values of R obtained by most other workers are satisfactorily explained by the finding that the observed values of R depend on an unsatisfactory method of determining the value of R.
3. The observation that the erythrocyte haemolyses to almost the same extent in solutions of a variety of simple salts shows that haemolysis depends on the extracellular osmotic pressure.
4. The erythrocyte haemolyses to exactly the same extent at different temperatures provided that the extracellular osmotic pressure is kept constant, and this could happen only if the cell were a perfect osmometer.

5. Direct proof has been obtained that the erythrocyte swells to an extent which can be predicted from a knowledge of the extracellular osmotic pressure - provided that one uses methods which do not involve centrifuging the artificially swollen cells.
6. It has been shown that the intracellular osmotic pressure of the erythrocyte is always the same as that of the surrounding plasma, under both normal and abnormal conditions (Table 34, p. 144).

These conclusions are all in the nature of simplifications of the behaviour of the erythrocyte towards its surroundings, but it must not be assumed that the red cell has been brought down to the level of a balloon which can be inflated or deflated at will. That part of cell behaviour which is related to osmotic swelling and osmotic haemolysis is subject to the laws of physical chemistry which are themselves neither simple nor complete.

The osmotic properties of the extracellular fluids of the body are easier to investigate at the bench than the properties of cells, but they are much harder to explain. The commonest body fluid, sent for biochemical examination, is plasma (or serum) and the analytical results are generally taken as an index index of the composition of the whole of the body's extracellular fluid. Without questioning the importance of serum analysis, it

must be admitted that its popularity depends wholly on the accessibility of blood. Yet there is no reason to believe that the chemistry of serum is more important than that of any other body fluid, and indeed it is known that the chemical composition of serum differs considerably from the composition of a fluid such as cerebrospinal fluid. Since all the various extracellular (and intracellular) fluids must traverse membranes before they can accumulate, chemical differences are inevitable.

The descriptions of osmotic and membrane effects in the human body receive essentially the same treatment in all standard textbooks and are based on the original observations of van't Hoff and Donnan. Although their original conclusions remain unchallenged in the field of physical chemistry, the applications of their work to the study of body fluids in living systems leave something to be desired. The chief stumbling blocks are these.

In all theoretical studies of the osmotic pressure developed with artificial membranes, the assumption is made that the membrane is freely permeable to water but completely impermeable to a large molecular solute. Movement of water through the membrane from pure solvent to solution results in the creation of a mechanical pressure in the form of a column of fluid, and this fluid column exerts its mechanical pressure in a direction opposite (but equal in magnitude at equilibrium) to the

osmotic pressure. At equilibrium, there is pure solvent on one side of the membrane and a (diluted) solution on the other. The freezing points (or osmolarities) of the two equilibrium fluids will therefore be quite different. This state of affairs is not found in the human body, and there is no body fluid which has zero osmolarity.

In all studies of the colloid osmotic pressure of large molecules such as protein, the assumption is again made that the membrane is freely permeable to water and to small molecules and ions, but impermeable to the protein molecule. The condition is possible in the laboratory, and, indeed, is the basis of all reliable methods of determining the colloid osmotic pressure experimentally, but very few body membranes are completely impermeable to protein; the normal renal glomerular membrane is probably the only one. It will be seen from the data in Table 35 (p. 150) that many fluid accumulations contain high concentrations of protein, approximating, in some cases, to the protein concentration of the corresponding serum. This raises the question of possible mechanical damage to membranes which are "normally impermeable" to protein. In this series, the only membrane separating plasma from the accumulated fluid, which is unquestionably "normal", is the membrane covering the choroid plexus through which the cerebrospinal fluid is formed. Since all the other fluid accumulations are abnormal, the

possibility is evident that the membranes through which they have accumulated are themselves abnormal. On the other hand, if the matter were merely one of straightforward mechanical damage, the chemistry of the accumulated fluid should be the same as that of the serum in all cases, and this is not so. To what extent mechanical damage may be a factor, is unknown, but it is certainly not important.

It is believed that all extracellular fluids are derived primarily from the blood plasma and it would be difficult to visualise any other source of origin. In order to reach its final location, the fluid must pass through the membrane of the capillary wall and then through the membrane surrounding the cavity in which it collects. All the fluids studied here (with the single exception of oedema fluid) occur either in potential body cavities or in loculated spaces. The protein composition of these true body fluids is different from that of the corresponding plasma: seldom, for example, do they contain any appreciable concentration of fibrinogen, and synovial fluid has a high concentration of mucoprotein. Taylor, Kinmonth and Dangerfield (1958) found an increased albumin:globulin ratio (compared with serum) in oedema fluid from cases of lymphoedema - the large α_2-globulins being greatly diminished in the oedema fluid. Spak (1960), however, has shown that ascitic fluid contains all the globulin fractions found in serum, whatever the cause of the ascites.

Many other observations of like character are to be found in the literature. In these fluids with an appreciable protein concentration - hydrocele, pleural, synovial and ascitic fluids - the albumin: globulin ratio of the fluid is approximately double the albumin:globulin ratio of the corresponding serum, presumably due to the relative ease with which the smaller albumin molecule can pass through membranes. This suggests some degree of passive transfer of protein governed by pore and molecular sizes. The important point is that, in all such cases, any conclusions based on the assumption that the membranes are "impermeable to protein" must be inadequate.

It is also generally believed that the colloid osmotic pressure due to protein is one of the main factors governing the movement of water between the various fluid compartments of the body. Although it may be a factor in certain cases and under certain undefined circumstances, there must be other more important agencies involved. This has been accepted in the case of cerebrospinal fluid: in its formation, fluid moves from the blood plasma into the subarachnoid space at a rate of up to 500 ml. per day. Were protein osmotic pressure the only factor, or even the dominant factor, fluid would move in the opposite direction. It does not do so for at least two reasons, (a) the blood has a greater hydrostatic pressure than the cerebrospinal fluid, and (b) work

is done by the membrane covering the choroidal plexuses in the ventricles. The same argument applies to all other fluids; were colloid (i.e., protein) osmotic pressure the governing factor, none of these fluids could ever accumulate.

An even better example is the maintenance of a constant volume by the circulating erythrocyte. The human erythrocyte has been shown to be a perfect osmometer which responds, short of haemolysis, to changes in the osmotic pressure of its environment in a predictable fashion. The colloid osmotic pressure within the erythrocyte due to the haemoglobin (mol. wt., 66,700 and concentration 34 g. per 100 ml.) is some 6 times the colloid osmotic pressure of the surrounding plasma, 90% of which is due to albumin (mol. wt., 65,600 and concentration 4.7 g. per 100 ml.). Yet in spite of this difference, the erythrocyte succeeds in maintaining its normal volume within very narrow limits of variation because it is capable of maintaining the same osmotic pressure on both sides of its membrane under both normal and abnormal osmotic conditions (Table 34, p. 144).

In all calculations based on the Donnan theory of membrane equilibrium, not only is the assumption made that the membrane is impermeable to large molecules, but the further assumption is made, or implied, that the fluid volumes are the same on both sides of the membrane, and remain the same, - e.g.,

Davson and Danielli (1952, p. 27), and many others. On the other hand, in clinical practice, the volumes of fluid on either side of a body membrane are never the same and are capable of violent fluctuation. The plasma volume of the normal healthy adult is about 3.5 litres and the volume of fluid in the normal peritoneal cavity is believed to be about 5 ml. In an average case of ascites, the volume of the ascitic fluid is commonly of the order of 5-10 litres and may be considerably more in longstanding cases of cirrhosis. It is also known that several litres of ascitic fluid can accumulate in the course of a few days. Fiese and Thayer (1950) removed 21.9 litres of oedema fluid in 10 hours from one patient by means of Southey's tubes, and they claim, with some considerable justification, that this constitutes a record. Such volume differences and fluctuations play havoc with theoretical calculations.

In studies of the Donnan membrane equilibrium, all <u>in vitro</u> experiments and calculations are based on the existence of only two forces which are equal and opposite at equilibrium - the osmotic pressure and the hydrostatic force which it generates. No doubt such forces exist also in the human body, but there are others as well. Starling recognised this in 1896 and equated four variables:-

"..... serum protein osmotic pressure <u>minus</u>

"tissue fluid protein osmotic pressure <u>equals</u>

"capillary hydrostatic pressure minus tissue "hydrostatic pressure."

Even this equation does not cover all the possibilities. The membrane of the choroid plexus is capable of performing work, and it also seems probable that the membrane surrounding a spermatocele can do the same. In one type of case, the accumulation of oedema fluid, the force of gravity determines the site of the fluid collection. This is of both clinical and biochemical importance, and the following case is an instructive example.

> The patient was a boy of 15 with acute nephritis, oliguria, and oedema. The blood chemistry was consistent with the clinical picture. When nursed in bed in the customary flat position, he developed epileptiform fits due to cerebral oedema. The fits stopped when the head of the bed was sufficiently raised, and he then developed marked sacral oedema. When his position was readjusted so that his legs were dependent over the end of a special bed, the oedema fluid gravitated to the ankles where it was removed by means of Southey's tubes. Several litres were removed in the course of a few days (Case no. 12 in Table 35).

In addition to tissue hydrostatic pressure, the whole mass of fluid in an enclosed cavity must also be under pressure of the surrounding musculature. Ascitic fluid is under a considerable pressure from

the muscles of the anterior abdominal wall, and the greater the distention, the greater the opposing muscular and elastic forces. Looked at from the opposite viewpoint, there are unknown forces concerned in the reabsorption of abnormal fluid collections. It is not in every case that the fluid has to be removed by needle or cannula: absorption of the fluid is often "spontaneous".

The colloid osmotic pressure is, by definition, pressure which can be exerted on only one side of a membrane; the substance responsible for it cannot traverse the membrane. The word "colloid" is introduced into the expression because one commonly associates impermeability with large colloid molecules. But this need not be so. H. B. Bull (1941) made the point very succinctly when he stated that:-

> "..... what an osmotic pressure measurement
> "does in essence is to indicate the number of
> "particles which cannot pass through a membrane
> "in a given weight of solution."

The particle size is irrelevant; so long as the particles do not pass through the membrane concerned, they will exert a "colloid osmotic pressure" on whichever side of the membrane they happen to be.

For a good example, we may again refer to the erythrocyte suspended in its native plasma. The normal concentration of potassium in the plasma is about 5 meq. per litre. The intracellular

concentration of potassium is about 100 meq. per litre. It can be argued that this difference is maintained by an ionic pump; that it is a net result in a dynamic system with potassium ions passing through the cell membrane in both directions; that it is maintained by the energy derived from glycolysis; and that the intracellular potassium can (and does) escape easily if this source of energy fails, as in a severe diabetic ketosis, and so on. Yet when all these arguments have been admitted the fact still remains that, at any given moment, the potassium concentration within the cells is some 20 times the plasma concentration, and the intracellular potassium gives rise, both in theory and in practice, to a "colloid osmotic pressure". This "colloid osmotic pressure" is balanced in such a way that the <u>total</u> extracellular and intracellular osmotic pressures are always equal (Table 34).

These facts lead to the conclusion that colloid osmotic pressure due to the presence of protein is not the governing factor, and indeed may not even be an important cause, in the movement of water between the various body compartments or in the accumulation of abnormal collections of body fluids. The movement of water may be governed with equal probability by the presence of small molecules and ions.

The primary cause of abnormal collections of body fluids remains to be discovered. Colloid osmotic pressure and protein concentration are not

the primary cause. Increased capillary permeability and mechanical damage to membranes are neither the cause nor important factors; if they were, the chemical analysis of all these fluids would give at least approximately the same results, whereas in fact, there are wide divergencies among the actual results recorded. This leaves two possibilities (a) failure of normal reabsorption of fluid and (b) increased membrane activity. In either case, the primary cause must be in the membrane and is still unknown. These membranes, through which the fluid must pass to reach its final destination, are not inert sheets of tissue. The data in Table 35 demonstrate that, in many cases, passage through the membrane produces a considerable change in chemical composition, showing that work has been done at the membrane. Osmotic equilibrium is established by movement of water following the osmotic gradient. The data given here indicate that osmotic equilibrium is not effected by the movement of small molecules such as urea, for in all cases, the urea concentration is almost exactly the same in the body fluid as in the corresponding plasma. The average difference between the concentration of urea in the fluid and in the corresponding plasma is a little less than 4 mg. per 100 ml. and several of the cases showed an abnormally high urea concentration.

The important conclusion deduced from the results presented here is that any accumulation of a

true body fluid is isosmotic with the corresponding plasma, and therefore, that all true body fluids are isosmotic with each other in any given individual - normal or abnormal. The law governing the movement of water between the body compartments is that the total osmotic pressure shall be the same on both sides of the membrane, irrespective of the chemical composition of the fluids.

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